Molecular markers for abalone research

by

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Declaration

Molecular markers for abalone research

I hereby declare that:

a) The above thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgment is made in the text of the thesis. Any other assistance is stated below:

My colleagues, Messrs Neville Sweijd (Department of Zoology, University of Cape Town), Jason Bartlett (CSIRO Marine Research) and Nick Elliott (CSIRO Marine Research) contributed to the development of the species identification technique presented as Chapter 8. Dr. Sweijd had previously developed a protocol for the identification of four abalone species utilising interspecific variation within the sequence of the lysin gene. Our aim was to extend this protocol to cover 11 species of commercial importance in the southern hemisphere. The approach taken to achieve this aim was conceived by a close collaboration between Dr. Sweijd and myself. The development of the protocol, and my role in its design and completion is presented as a prelude to Chapter 8. The validation of the inter-specific nucleotide differences identified by Dr. Sweijd and myself was undertaken by Mr Bartlett, who tested the protocol on large numbers of samples. The manuscript submitted for publication was written by Dr. Nick Elliott in close collaboration with myself.

My colleague, Dr. Neville Sweijd assisted me in the collection and processing of abalone samples in South Africa (Chapter 6). The conceptualisation and development of the methodology and subsequent analysis and interpretation of the data set was conducted independently by myself.

My colleagues, Jason Bartlett and Natalie Conod assisted in the collection of data for chapter 4. This data was collected using markers that I have developed (chapter 2), and all subsequent analysis and investigation of the data was conducted independently by me.

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Abstract

Molecular markers for abalone research
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The Haliotidae is a family of marine gastropod molluscs, of the Order Archaeogastropoda (Schremp 1981). The world-wide family consists of 56 currently described species from temperate and tropical waters of both hemispheres. Approximately 25 abalone species are currently harvested commercially from at least 15 countries, with many more taken by recreational fisherman.

This thesis examines the utility of molecular genetic markers in abalone research, with particular emphasis to the Australian fishery and aquaculture industries. The development of microsatellite markers in temperate abalone, and their use in stock structure studies of abalone fisheries in Australia and South Africa are presented.

Twenty-two microsatellite loci were isolated from a *Haliotis rubra* partial genomic library, and their conservation in 12 other *Haliotis* species is presented. A maximum of 15 of these markers were retained in the most closely related species, *Haliotis conicopora*, but the species status of this group is questioned. Only 3 of the microsatellite loci examined were retained in each of two North American species, *Haliotis corrugata* and *H. fulgens*. Methods for the detection of microsatellite loci in other species is also discussed.

A study of genetic variability in *H. rubra* at eight microsatellite loci in seven Tasmanian, one Victorian and two New South Wales locations is presented. These locations represent both fine (5 km) and broad (> 500 km) scale separation, and reveal a mean H_e of 0.552, with significant genetic differentiation between Tasmanian samples and those from mainland Australia (F_{CT} = 0.003; $P < 0.0001$). No significant differentiation was identified between Tasmanian samples, or between mainland samples. Significant departures from Hardy-Weinberg equilibrium were common, in all instances due to an excess of homozygotes. Evidence for the existence of null alleles at two loci is presented as a factor in the departures from H_e.

In this thesis I also examine the utility of microsatellite markers developed in *H. rubra*, to investigate the structure of *H. midae* populations in South Africa. Variation at three microsatellite loci was examined at six locations and showed a mean H_e of 0.528, and revealed a significant decline in the number of alleles from western to eastern samples (West N_{allele} = 12.3; East N_{allele} = 7.7). Highly significant differentiation was observed between samples from the west and east of Cape Agulhas (F_{CT} = 0.033; $P < 0.0001$). The results are discussed and compared to the findings of two previous studies and possible explanations are provided for the apparent contradiction between the allozyme data and that provided by mitochondrial DNA and microsatellite variation.

Microsatellite DNA markers were also used to investigate genetic diversity within cultured populations of *H. rubra* and *H. midae* in Tasmania and South Africa respectively. All cultured populations examined were the product of wild caught broodstock, and show a loss of genetic diversity, measured as number of alleles per locus, but no associated loss of heterozygosity. The production of 3 *H. rubra*, *H. laevigata* hybrid family lines is outlined, and evidence for selection against a microsatellite allele in one of those family lines is presented.

The global problem of abalone poaching is also discussed, and a DNA based PCR-RFLP protocol for the identification of 11 southern-hemisphere species of abalone is presented. The technique utilises variation within short (< 200 bp) fragments of the mitochondrial COI and COII genes, and is simple to perform in most standard genetic laboratories.

This thesis presents a discussion of the influence of molecular genetic techniques on both wild fishery and aquaculture of abalone around the world, as well as providing some direction for the future.

Acknowledgments

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I thank Boze Hancock of Fisheries Western Australia for samples of *H. roei and H. conicopora*, Liz O'Brien of the University of Queensland for samples of *H. asinina*, Neville Sweijd of the University of Cape Town, South Africa for samples of *H. midae* and *H. spadicea*, Phil Critchlow for samples of *H. scalaris*, Miguel Angel Del Rio of CICESE in Ensenada, Mexico for samples of *H. fulgens* and *H. corrugata*, and Rodney Roberts of the Cawthron Institute, New Zealand for samples of *H. iris*, *H. virginea* and *H. australis*.

I thank Pete Cook, Neville Sweijd and Collen O'Ryan for looking after me at UCT and Genee Harms for the use of the ABI-373 at Groote Schur Hospital, Cape Town. I would also like to thank all the people who made my stay in South Africa so productive and enjoyable, particularly Maria Stacey and Neville Sweijd for taking me into their home and making me feel so welcome. To Verna and all the guys and girls from the Fitz, thanks for everything.

Special thanks must be reserved for the management and staff at the Tasmanian abalone hatchery who led me by the hand through the spawning, settlement and on-growing of abalone for research, whilst still maintaining their commercial focus

I thank Bob Ward, Dan McGoldrick, Sharon Appleyard, John Benzie, Adam Smolenski, Simon Jarman, Bronwynn Innes, Cath Sliwa, Jason Bartlett, Stan Robert and Peter Rothlisberg for helpful comments on papers arising from this thesis, and for being available to discuss my research and the many small difficulties that arose throughout its course. I thank Alice Morriss for her Excel expertise that I blatantly borrowed to make my life easier this past year.

To my friends, all of whom have been very forgiving of someone who has serially declined social invitations of late, and has made no real efforts to keep in contact. Your support (and the home cooked meals) has been very much appreciated and I will attempt to make it up to you all in the coming months and years. Thanks also to Doiv and the staff at knoppies for supplying an avenue of escape when required.

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track, and his continued enthusiasm for whatever path I chose was essential, and much appreciated. Your friendship was as important as your supervision, thank you Nick.

My family also deserve special praise, their emotional and financial support has been amazing throughout my research, they have always believed in me, despite my own concerns, and are always there for me. Mum, thanks for the long walks, talks and many hot dinners. Dad, thanks for wanting to spend time on the water with me, I'm looking forward to many days of relaxing boating to make up for the days we've missed. Michelle, thanks for being such a great sister to an average brother, typified by the wonderful gift of seeing John's last game, and another Wallaby triumph during such a draining time, it really helped.

This PhD has taken me through hard times and wonderful times, it has made me wonder if I could, and decide that I can. Through this work I met my wonderful girlfriend Carryn, but also because of this work I have been away from her for too long. The end of this thesis marks a new beginning for us both.

At last!

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Chapter 1 Abalone and molecular markers

1.1 Abalone, Genus *Haliotis*

Abalone are a family of marine gastropod molluscs, of the order Archaeogastropoda (Schremp 1981). The world-wide family consists of 56 currently described species from the temperate and tropical waters of both hemispheres (Geiger 2000). Early research identified a number of genera based on shell form, but this taxonomy was questioned by Cox (1960) who recommended all species within the family Haliotidae be classified into the single genus *Haliotis* until more definitive taxonomic evidence for separating genera became available. Species delineation has been described as tenuous (Brown 1991), and is blurred further by the existence of natural hybrids between morphologically distinct species (Brown and Murray 1992). Geiger (2000) has recently reviewed literature and numerous specimens to produce the most current taxonomic description of the family, but he cautions that these groupings should be used only until a comprehensive phylogenetic analysis of the family is available.

1.2 Harvesting a resource

It is likely that abalone have been harvested by man as a food source for centuries. Geological records in southern Africa suggest that the South African abalone or Perlemoen (*Haliotis midae* Linnaeus 1758) was utilised by humans as long as 125 000 years before present (Voigt 1988). Evidence from Australian aboriginal middens (a mound consisting of shells of edible molluscs and other refuse, marking the site of prehistoric human habitation) show that abalone have been a significant part of the diet of coastal dwellers since humans first colonised Australia approximately 40 000 years ago (Bailey 1975). Abalone has been an important resource in many ancient cultures, both directly as a foodstuff and indirectly through the trading of shells. The exploitation of abalone as a commercial resource is a relatively recent activity and is of importance in all of Australia's southern states, and in many countries around the world.

Approximately 25 abalone species are currently harvested commercially from at least 15 countries (FAO 1999). Many more species are harvested worldwide on a recreational basis using both traditional skindiving, and modern SCUBA and surface supply methods. In 1999 the total commercial abalone harvest reported worldwide exceeded 10 000 t (FAO 1999), not including those taken by recreational divers and subsistence fisheries. The amount of abalone taken by illegal means is not known, but estimates range from 10 to 100% of the legal catch (Personal communication; Neville Sweijd, University of Cape Town, Cape Town). Australia is a major producer of wild harvested abalone (5 508 t in 2000, ABARE 2001), and by far the

largest proportion of that harvest is from the Tasmanian abalone (*Haliotis rubra* Leach 1814; *H. laevigata* Donovan 1808) fishery (2 565 t in 2000, ABARE 2001).

In addition to the wild fishery for abalone, there is an established and expanding commercial interest in the culture of the genus in at least 10 countries. The major producers of cultured abalone are Japan, China, Taiwan, with rapidly increasing production occurring in Australia, South Africa, Mexico and the USA (FAO 1999).

Abalone is currently marketed primarily in eastern countries with the majority of the wild and cultured product being exported to Japan (84% in 1993-94) and Hong Kong (13% in 1993-94). In 1999-2000 approximately 70% of the total Australian production of abalone was exported as either canned product (2 118 t, ABARE 2001) or fresh, chilled or frozen (1 691 t, ABARE 2001).

1.3 History of abalone research

Carl Linnaeus (Carl Von Linné) provides the earliest record of haliotid research in *Systema Naturae* (1758), which contains the original descriptions of many abalone species. Linnaeus also facilitated the publishing of Gmelin's descriptions of New Zealand abalone species in a later edition of the same monograph (Linné 1791). Following these early descriptions, the first biological studies of abalone were produced by the French anatomists Lacaze-Duthiers in 1859 and Wegmann in 1884, and then by Croft in 1929. By far, the majority of haliotid research has been produced in the late twentieth century and beyond. Since the early 1970s the level of research into various abalone species has increased exponentially, with most concerned with the basic biology and ecology of abalone species. Research interests have mirrored commercial interests in that time, with the majority of research being directed to species of high commercial value, and the investigation of traits important for the sustainability of the fishery as well as the ecology of the species.

Early research examined such parameters as age at maturity (Shepherd and Laws 1974), growth rates (Hirose 1974), reproductive success (Rho and park 1975), recruitment and mortality (Shepherd 1986), population density (Breen and Adkins 1982), size structure of fished and non-fished populations (Adkins and Stefanson 1977), larval settlement and dispersal (Tegner and Butler 1985) and predator-prey dynamics (Shepherd 1973). These areas of research have been examined in many species because of their importance to the health of lucrative abalone fisheries (worth over $235 million in Australia, 1999-2000, ABARE 2001). Ongoing research in these areas ensures that abalone stocks are managed in a sustainable manner.

In more recent times, the focus of abalone research has shifted towards the conservation of declining abalone resources (Shepherd and Rodda 2000; Shepherd *et al.* 1998), and to areas more directly related to the expansion of abalone culture (Capinpin *et al.* 1999; Friedman *et al.* 2000). As the abalone culture industry expands, so does the need for specific information on spawning, fertilisation, hatching and grow-out of the animals. Diet, water quality and tank design are also areas of research that have seen substantial efforts in recent times.

An example of the shift in research focus can be seen in a review of the papers presented at the 4th International Abalone Symposium held in Cape Town, South Africa (Feb 2000). At this symposium, at least half of the oral presentations covered topics of commercial interest to aquaculture, including larval settlement, diet preference, physical tolerances, growth rates, survival and the evaluation of shore and sea-based culture systems. This is in stark contrast to the 1st International Abalone Symposium held in La Paz, Mexico in 1989, where only 10 aquaculture related papers were presented (of which four were regional reviews) out of a total of 46 papers.

1.4 Genetic research

The influence of genetic research on abalone industries has likewise increased dramatically as molecular techniques have improved and research focus changed. Prior to the initiation of my research thesis, only seven genetic studies of abalone were published (Fujio *et al.* 1983; Barrett 1989; Brown 1991; Brown and Murray 1992; Jiang *et al.* 1995; Mgaya *et al.* 1995; Gaffney *et al.* 1996). Since the inception of this research in 1997, there has been a proliferation of research articles presented, with no less than 14 studies being published, or currently in press. The most dramatic increase in publications occurred in the year 2000 with the presentation or publication of seven studies (Kirby *et al.* 1998; Sweijd 1999; del Río Portilla 2000; Hamm and Burton 2000; Zúñiga *et al.* 2000; Gutiérrez González 2000; Hancock 2000; Burton and Tegner 2000; Chávez 2000; Conod *et al.* In Press; Selvamani *et al.* 2000; Huang *et al.* 2000; Evans *et al.* This thesis; Evans *et al.* In Press). It is likely that the rapid increase in the number of genetic studies of wild abalone fisheries will subside as the genetic structure of commercially important species is better understood. It is also likely that the decreasing number of wild fishery studies will be offset by an increase in the amount of research into aquaculture genetics. Genetic parameter estimations and other quantitative genetic studies such as marker assisted selection programs are likely to increase as the abalone culture industry follows the path of more established industries such as livestock production and the salmon, tilapia and prawn culture industries (Mosig *et al.* 2001; Danzmann *et al.* 1999; Moore *et al.* 1999; Sakamoto *et al.* 1999). Molecular genetics in areas such as gene expression and genetic engineering are already underway and will become more prevalent in the future (Morse 1996; Powers 1995; Wodicka and Morse 1991).

Development of genetic markers in abalone research has, as expected, mirrored their application in other fields. Most genetic investigations of abalone examine population level variation at protein coding allozyme loci. The aim of these studies was to provide a better understanding of the reproductive structure of abalone stocks, so the relevant fishery could be managed at a sustainable level. Early research was often hampered by the suggestion that allozymes were not variable enough to reveal genetic differences between populations that had only recently become reproductively isolated. Advances in genetic technologies have since provided more variable markers such as mitochondrial DNA (mtDNA) and hyper-variable nuclear DNA markers such as microsatellites that are fast becoming the marker of choice for population level analyses (see Ward and Grewe 1995). All these genetic techniques have been trialed in abalone with mixed results, and there are now a number of options available to the abalone researcher.

1.4.1 Uses in wild fishery

The use of genetic markers in wild fisheries has been a traditional area of genetic research. There have been numerous studies investigating the stock structure of marine species ranging from large pelagic vertebrates (eg. Chow *et al.* 1997) to sessile invertebrates (eg. Apte and Gardner 2001). Genetic markers have been used to examine the population structure of 12 abalone species. Twenty-one studies of abalone stock structure have been published in the past 20 years (Table 1-1), 13 of which measured genetic variation at protein coding allozyme loci. Five studies have examined mtDNA variation, and recently the microsatellite has become the marker of choice to investigate stock structure in abalone species, with six studies since 1998 examining variation at these highly polymorphic loci (this figure includes Chapters 4 and 6 of this thesis). One of these studies (Huang *et al.* 2000) also investigated the utility of randomly amplified polymorphic DNA (RAPD) and minisatellite DNA variation in the blacklip abalone, *Haliotis rubra*. The investigation of stock structure in abalone, as with other commercially harvested species has been driven by the need for biological information in order to ensure that the fishery is harvested at sustainable levels. Knowledge of the stock structure of abalone is one of a number of important tools available to fishery managers to achieve this goal. The level of genetic differentiation detected by these studies varies greatly, and is a factor of the genetic marker examined and the biology of the species. The genetic markers listed earlier differ in their power to detect stock differentiation. These differences can be attributed to various factors associated with the mutation rate and effective population size of the marker. Chapters 4 and 6 detail differences in the power of genetic markers and their suitability for detecting population structure in abalone.

Table 1-1 Studies of genetic variation within abalone populations

Marker	Species	Researchers
Allozymes		
	H.discus hannai	Fujio *et al.* 1983
	H.rubra	Brown 1991
	H.laevigata	Brown and Murray 1992
	H.tuberculata	Mgaya *et al.* 1995
	H.rufescens	Gaffney *et al.* 1996
	H.midae	Sweijd 1999
	H.corrugata	del Río Portilla 2000
	H.cracherodii	Hamm and Burton 2000
	H.fulgens	Zúñiga *et al.* 2000
	H.fulgens	Gutiérrez González 2000
	H.roei	Hancock 2000
	H.rufescens	Burton and Tegner 2000
	H.rufescens	Chávez 2000
mtDNA		
	H.rubra	Barrett 1989
	H.diversicolor	Jiang *et al.* 1995
	H.midae	Sweijd 1999
	H.cracherodii	Hamm and Burton 2000
	H.rubra	Conod *et al.* In Press
microsatellite		
	H.rufescens	Kirby *et al.* 1998
	H.asinina	Selvamani *et al.* 2000
	H.rubra	Huang *et al.* 2000
	H.rubra	Conod *et al.* In Press
	H.rubra	Evans *et al.* This study
	H.midae	Evans *et al.* In Press
RAPD		
	H.rubra	Huang *et al.* 2000
minisatellite		
	H.rubra	Huang *et al.* 2000

1.4.2 Uses in enforcement

In recent years, the effect of abalone poaching on stock densities and the economic impact on legal fishers has been highlighted by the work of Hauck (1997) and Sweijd (1999) in an examination of the South African abalone fishery. The work in that country has shown that high levels of poaching are organised by sophisticated syndicates who procure, process (dry and canned abalone) and illicitly export the abalone to the Far East. In South Africa, Australia, New Zealand, Mexico, the USA and Canada, abalone poaching has been identified as a problem to varying degrees (see Sweijd 1999 for review). The over-exploitation of these resources means that many abalone stocks are in decline. In the USA at least one species,

Haliotis sorenseni (Bartsch 1940) is listed as commercially extinct (Tegner 1993; Tegner *et al.* 1996; Davis *et al.* 1998), resulting in the closure of the commercial fishery for that species.

Traditionally, valuable fisheries have been protected through the use of closed seasons or areas, size or gear restrictions and catch limitations. These methods of protection have also been employed in most abalone fisheries around the world, but as in every fishery, the protection is only effective if it can be enforced. The problem faced by many abalone fisheries is the lucrative profits that can be made illegally from the species, leading to more organised efforts by poachers. Whilst there is no substitute for traditional enforcement methods, genetic markers may provide necessary evidence in cases where the identity of the product is in dispute. Genetic markers have previously been used to identify the substitution of endangered fish species for a more common variety (De Salle and Birstein 1996). Recent work has used similar methods to discriminate 11 Southern Hemisphere species of abalone (see Chapter 8), and it is likely that development of genetic identification techniques will proceed rapidly in the coming years, with particular emphasis on commercially valuable marine species.

1.4.3 Uses in aquaculture

The use of genetics, and particularly molecular genetics, in recent years to increase quality and yield in such industries as livestock and plant production, has become standard practice. Genome mapping and the identification of quantitative trait loci (QTLs) using DNA markers is widespread (beef: Moody *et al.* 1997; crops: Mohan *et al.* 1997). The application of selective breeding technology to the improvement of aquaculture species has not been so rapid. Reasons for the slower adoption of genetic improvement programs in aquaculture include a lack of maturity of industries and a misunderstanding of the potential economic benefits of initiating breeding programs. This late start has resulted in a limited number of molecular genetic markers available for cultured species, marine invertebrates such as abalone in particular. Because biological traits for all living organisms are under the influence of genes, the same genetic technologies that have been used to improve livestock can be used to improve aquaculture species. The few aquaculture improvement programs conducted to date have yielded impressive results (Bondari 1983; Gjerde 1986; Dunham 1987) and demonstrate that there is enormous potential to dramatically improve productivity of aquaculture systems through selective breeding.

There are five species of abalone presently under culture in Australia: the greenlip (*H. laevigata*), donkeys ear (*H. asinina* Linnaeus 1758), Roes (*H. roei* Gray 1826), staircase (*H. scalaris* Leach 1814) and blacklip abalone (*H. rubra*). Hatcheries presently collect the majority of their broodstock from the wild, but are becoming increasingly reliant on cultured broodstock. Commercial hatchery spawnings currently occur outside of any controlled

breeding programs and usually involve multiple individuals of both sexes with little regard for which parents are successful. The application of DNA markers to pedigree analysis within such spawnings would ensure that the number of contributing adults is known and any likelihood of inbreeding in hatchery stocks is highlighted.

The development of molecular DNA markers in abalone species facilitates the use of marker assisted selection in aquaculture. Markers can ensure that broodstock collected from wild populations or produced in family lines are more likely to produce gametes and larvae with improved survival, faster growth and other desirable characteristics than those chosen purely on the basis of gonad size. The use of genetic markers to assist in producing improvements in quality and yield is generally accepted by the consuming public as the animals are not genetically modified, but simply genetically selected. Molecular DNA markers can also be used to assess the success or impact of stock enhancement programs such as those currently underway in New South Wales, Australia (Fletcher 1999), and Cape Town, South Africa (Sweijd 1999).

Research to improve the diet and grow-out systems of cultured abalone has been underway for several years (eg Uki *et al.* 1986; Oakes 1988). Growth in abalone is reported to be closely related to genetic factors (Hara 1990; Mgaya and Mercer 1995). If environmental improvements can be achieved in conjunction with improved broodstock selection, facilitated by the use of molecular markers, then growth rates could be increased by as much as 10 to 20% per generation (Oysters, 21% - Bondari 1983; Atlantic salmon, 14.4% - Gjerde 1986; Channel Catfish, 20% - Dunham 1987). Associated with increased growth rates is often an increase in feed conversion efficiency, so it costs less per animal to farm, in turn leading to greater productivity and profitability for the industry.

Although large-scale selection programs are initially expensive to conduct, the long term cost benefits demonstrate that the returns from aquaculture breeding programs are equal to or better than from livestock. The Norwegian Atlantic salmon breeding program for example, estimates a cost-benefit ratio of 1:15. Therefore, selection programs in the long-term have proved very cost effective and should be considered carefully for the future of the abalone culture industry.

1.5 Summary of contents

This thesis enhances the utility of molecular genetic markers in abalone research, with particular emphasis on their benefits to the Australian fishery and aquaculture industries. The development of a new class of molecular marker in temperate abalone species, and their use in examinations of abalone fisheries in Australia and South Africa are presented. The enhancement of abalone aquaculture and species identification using molecular genetic techniques is also investigated.

Chapter 2 details the development of a suite of new microsatellite markers in the Tasmanian blacklip abalone, *Haliotis rubra*. This chapter is published as Evans *et al.* (2000).

In chapter 3, the conservation of 22 microsatellite primer pairs in another 12 abalone species from around the world is investigated. The chapter includes a discussion of the way in which marker conservation has been reported in the past, and provides insight into the utility of these markers in other commercially important abalone species. This chapter is published as Evans *et al.* (In Press).

Chapter 4 is an examination of genetic variation at eight microsatellite loci in the blacklip abalone, *H. rubra* from ten sites in Tasmania and mainland Australia. Evidence for limited differentiation between Tasmanian and mainland Australian stocks is presented, and compared to previous allozyme and microsatellite examinations. Implications of the data are discussed in the context of fishery management.

Chapter 5 identifies the presence of null alleles at two microsatellite loci. Evidence is presented of point mutations in the primer site(s) of two individuals, and preferential amplification in another individual at one locus. Inefficient amplification is discussed as a causative agent for null alleles at another locus.

Chapter 6 examines the transfer of microsatellite markers designed for *H. rubra* to an investigation of genetic variation in the South African abalone, *H. midae* around the South African coast. Evidence of a genetic discontinuity at three microsatellite loci in stocks of *H. midae* is presented and results discussed in consideration with previous mitochondrial DNA and allozyme data (Sweijd 1999). This chapter has been submitted to Marine Biology for publication.

In chapter 7, the genetic management of abalone farming is discussed, with particular reference to a single year class produced in one Australian and two South African abalone farms, as well as a number of experimentally produced *H. rubra*, *H. laevigata* hybrid abalone

"family lines". The maintenance of genetic diversity in hatchery breeding and the development of genetic breeding programs is discussed.

Chapter 8 details the development of a protocol for the identification of 11 species of abalone common to the Southern-Hemisphere. The chapter details the problems of abalone poaching in many countries and provides a new technique in the fight against illegal harvesting and processing.

Chapter 9 provides a review of the major findings of this thesis and presents a discussion of the influence of molecular genetic techniques on both wild fishing and aquaculture of abalone around the world, as well as providing some direction for the future.

Chapter 2 Characterisation of microsatellite loci in the Australian Blacklip abalone (*Haliotis rubra,* Leach)

2.1 Prelude

This chapter describes the process of the development and characterisation of microsatellite loci in *Haliotis rubra*. The manuscript presented below, appears as published in Molecular Ecology 9, 2000, and presents details of the first 9 loci that I developed for *H. rubra*. In total I developed PCR primers for 22 microsatellite loci, all of which are presented in Chapter 3, and published as Evans *et al.* (In Press).

2.2 Published manuscript

Evans B, White RWG and Elliott NG 2000. Characterization of microsatellite loci in the Australian blacklip abalone (*Haliotis rubra* Leach). *Mol Ecol* 9: 1183-1184

Abalone are gastropod molluscs of the genus *Haliotis,* a commercially valuable foodsource. Wild stocks are harvested throughout the world in temperate and tropical marine systems by recreational and commercial fishers. The high demand for abalone and the decline in many of the world's natural stocks has led to increasing aquaculture of this genus, and a concurrent increase in the need for genetic information. *Haliotis rubra* forms the basis of the world's largest wild abalone fishery. In South-Eastern Australia it is the subject of an expanding aquaculture industry. Polymorphic microsatellite DNA markers in *H. rubra* are being developed principally for population structure analysis and for pedigree analysis, as well as future genetic mapping projects. Their development will be beneficial to both wild fishery managers and aquaculturists. I report here the isolation and characterisation of 9 microsatellite loci in *H. rubra.*

A partial genomic DNA library was constructed from DNA extracted from gill tissue of a single individual, using a modified CTAB method (Grewe *et al.* 1993). DNA was digested with *Sau*3A enzyme and the 500-700 bp fraction was ligated into the dephosphorylated *Bam*HI site of the vector *pGEM™-3Zf(+)* (Promega) (Reilly *et al.* 1999). Ligated plasmids were transformed into XL-1 Blue Supercompetent cells (Stratagene). Cells were plated onto selective media and replicated onto uncharged nylon membrane filters (Boehringer Mannheim).

This library was screened for microsatellite repeats with a $(CA)_8RT$ probe. The probe was 3' end labelled with digoxygenin, and standard hybridisation and wash conditions were used (Boehringer Mannheim 1995). The nucleotide sequence of 62 positive candidate clones was

determined with ABI Prism™ Dye Terminator Cycle Sequencing (Perkin Elmer) using double-stranded plasmid DNA, prepared by the alkaline lysis method (Sambrook *et al.* 1989). Sequencing products were analysed on an ABI377 DNA autosequencer. PCR primers were designed using the OLIGO®computer software (Rychlick 1996), with one primer from each pair labelled with a fluorescent dye. Primers are summarised in Table 2-1.

PCR reactions were performed in a volume of 25 μL consisting of 67 mM TrisHCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg.mL^{-1} gelatin; 2.5 mM $MgCl_2$; 10 pmoles of each primer; 200 μM dNTPs; 0.5 U *Taq* F1 polymerase (Fisher Biotech); and ~ 20 ng genomic DNA template. Amplification was in a Perkin Elmer 9600 thermocycler with one cycle of 94° C for 1 min, 52 - 58.5° C for 15 s and 72° C for 1 min, followed by 30 cycles of 94° C for 15 s, 50 - 56.5° C for 15 s and 72° C for 1 min. These cycles were followed by a final extension step of 72° C for 10 min. Amplification products were initially visualised on 2% TBE agarose gels. Then they were diluted relative to amplification strength, mixed with formamide, loading dye and Genescan Tamra 500 size standard (ABI), denatured at 95° C for 2 minutes, and loaded onto a 4% denaturing polyacrylamide gel. Samples were run on an ABI377 DNA autosequencer and genotypes determined with Genotyper® software. Observed and expected heterozygosity from at least 14 individuals are shown in Table 2-1.

The 9 microsatellite markers retained for use exhibit a range of variation. They will be used to examine stock structure of the blacklip abalone fishery, inbreeding levels in hatcheries and parentage analysis in hatchery reared abalone, a precursor to selective breeding programs.

Table 2-1 Blacklip abalone (*Haliotis rubra*) microsatellite primer sequences, repeat descriptions, annealing temperatures and expected allele sizes. Observed (H_o) and expected (H_e) heterozygosity are shown for each locus, based on at least 14 individuals (n). The clone sequences from which the primers were designed have GenBank accession numbers AF194951 to AF194954 and AF194956 to AF194960.

Locus	Repeat Sequence	Primer Sequence (5'-3') (F-Forward, R-Reverse)	n	Anneal Temp.	H_o	H_e	allele No.	Expected Size (bp)
CmrHr 1.11	$(AC)_{15}$	F-ACTTTTTGATAGCCCCTC R-GCTTAGATGAAAGCCTTAAC	14	50.0° C	0.43	0.41	2	172-176
CmrHr 1.14	$(GT)_{13}TT(GT)_2$ GA $(GT)_3$	F-CTACGTACACTTTAATGTGCTC R-CTGCCTAAAAGTTCAATCC	31	53.0° C	0.25	0.40	4	251-275
CmrHr 1.24	$(AT)_8$	F-TCTAGCATGTCTGAGGGAGG R-TGTGTCATTGTGGTCGAAAG	27	54.0° C	0.28	0.50	4	222-228
CmrHr 1.25	$(CA)_{25}(AT)_6TT$ $(AT)_5(TG)_3$	F-CATCTTGGCTGAACATTAC R-AACGAACTCATTTCAGAGAC	14	54.0° C	0.14	0.83	9	291-309
CmrHr 2.9	$(GT)_{27}$	F-TAGCAGCCATAGGGTGGGTC R-CTGAATGGGGCTAGCACAAT	14	56.5° C	0.43	0.87	13	159-233
CmrHr 2.14	$(GAGT)_{8...}$ $(GAGT)_5$	F-GTCCTCCAGTGAGACCCAAA R-AGCATGGGTATTGTTGACTG	17	55.5° C	0.76	0.79	8	199-237
CmrHr 2.26a	$(ATTC)_5T_4C$ $(ATTC)_2$	F-TTCGGACTACAATCTGGAGGA R-CAACCTCAAACCGCATCTTT	15	55.5° C	0.60	0.83	8	190-212
CmrHr 2.30	$(GT)_6..(GT)_{13}$ $(TG)_{12}(AG)_5$ $.(TG)_3.(TG)_{16}$	F-TTGGCAGTGATGGAAACTTG R-TTCCAAACTGACACAGACGC	16	55.5° C	0.60	0.90	16	284-328
CmrHr 2.36	$(AC)_{21}$	F-CACCCTTTGGCATGAAAGAT R-ACCAACAGGGGCAGATACAG	15	56.5° C	0.46	0.74	8	83-121

Chapter 3 Evaluation of microsatellite primer conservation in abalone.

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3.1 Introduction

Members of the genus *Haliotis*, commonly called abalone, are distributed in coastal waters of all continents. Many of the 56 recognised species (Geiger 2000) are harvested commercially or recreationally, and they are a highly valuable marine resource. Abalone populations, like those of most highly prized marine resources, have come under increased legal and illegal harvesting pressures in recent years as demand for the product continues to rise, and methods for their capture and distribution are refined. Along with the expanding effort within abalone fisheries worldwide, and in some cases the decline of those fisheries, there has been extensive development in the culture of many abalone species (Oakes and Ponte 1996; McBride 1998; Cook 1998). An important technological advancement that will benefit both the culture and wild harvest industries is the development and application of molecular genetic markers.

Molecular genetic markers are widely used in many seafood industries for both wild and aquaculture needs; for example, in salmonids (O'Reilly *et al.* 1998) and oysters (McGoldrick and Hedgecock 1996). They can be used for applications as diverse as tracking the biological history of populations (Chambers and MacAvoy 2000), or as specific as determining the parentage of individuals in culture (O'Reilly *et al.* 1998). One such marker that has been applied in other genera (Dallimer 1999; Wu *et al.* 1999; Nesje *et al.* 2000) but has only recently become popular in abalone research is microsatellite DNA. Microsatellite markers consist of a nucleotide sequence of between 2 and 6 base pairs repeated in series at a set point on a chromosome (locus). The number of times that sequence is repeated at a single locus varies within (heterozygous individuals) and between individuals (intra-specific variation), and where that same locus is conserved across species, the number of repeats may vary widely (inter-specific variation) (Wright and Bentzen 1994).

Microsatellite markers have been developed from partial genomic libraries of five *Haliotis* species: *H. asinina* (Selvamani *et al.* 2000); *H. discus discus* (Sekino *et al.* 1999); H. *kamtschatkana* Jonas 1845 (Miller *et al.* 2000); *H. rubra* (Huang and Hanna 1998; Evans *et al.* 2000 - Chapter 2); and *H. rufescens* Swainson 1822 (Kirby *et al.* 1998). The development of microsatellite DNA markers, as described in each of these papers, is a time consuming and expensive process (Wright and Bentzen 1994). For these reasons, the efficacy of markers between species within the same genus or family have been examined in both plant and animal groups with ambiguous results (Rosetto *et al.* 2000; Huang and Hanna 1998).

White and Powell (1997) tested 11 microsatellite markers developed for the hardwood, *Swietenia humilus* for conservation within 11 members of the Meliaceae family, representing 7 genera. They detailed 4 species-specific, 1 genus-specific and 3 family-wide markers. This trend of good marker conservation within plant families is supported by other studies such as that by Thomas and Scott (1993) who found that primer sequence conservation existed among grapevine species, and more recently by Rosetto *et al.* (2000) who showed similar sequence conservation among members of the Myrtaceae family.

Conservation of 11 microsatellite loci developed for the Walleye, *Stizostedion vitreum* in four species representing two genera of the Percidae family was examined by Wirth *et al.* (1999) . Three of the markers were conserved in all species tested, two were found to be specific to *Stizostedion* genus, four produced amplification from both genera, but not all species within them, and the remaining two markers amplified only one other *Stizostedion* species. Primer sequences have also shown some conservation across 10 species of 4 genera of lemur, endemic to Madagascar (Jekielek and Strobek 1999).

Huang and Hanna (1998) considered the cross-species amplification of their 3 *H. rubra* microsatellite loci in species from USA (2 species), South Africa (2 species), South Korea (6 species) and Australia (5 species). Of the 10 species tested from outside of Australia, only two of the South Korean species produced any amplification product. Within Australian species the markers were more conserved, with at least two of the three loci producing an amplification product in all Australian species tested, except for *Haliotis laevigata*, the greenlip abalone, which failed to amplify a product at any of the 3 loci. As *H. rubra* and *H. laevigata* are known to produce hybrids in the wild (Brown, 1995) this latter result is unexpected, and required further examination as the hybrid is being developed as an aquaculture product for which molecular markers are required.

In this paper I describe the cross-species amplification of 21 microsatellite loci (22 primer pairs) developed for use in the Australian blacklip abalone, *Haliotis rubra* (Leach 1814). Twelve species from Australia (5 species), New Zealand (3 species), South Africa (2 species) and North America (2 species) were tested. These 12 species come from 2 discrete phylogenetic clusters within the genus based on sperm lysin DNA sequences (Lee and Vacquier 1995). I then expand upon this work by describing the optimization of some of these loci for genetic variation research in wild and cultured South African *Haliotis midae,* pedigree analysis and genetic variation research with the Australian *H. laevigata,* and genetic variation studies in the North American abalone, *H. fulgens* Philippi 1845.

3.2 Materials and methods

3.2.1 Microsatellite amplification.

The twelve abalone species tested in this study were chosen to provide both close and distant evolutionary relationships to *Haliotis rubra*. Four species (*H. conicopora* Péron 1816, *H. laevigata*, *H. roei*, *H. scalaris*) share a temperate Australian habitat with *H. rubra*, while *H. asinina* is a tropical species from Australian waters. *Haliotis australis* (Gmelin 1791), *H. iris* (Gmelin 1791) and *H. virginea* (Gmelin 1791) are temperate species from neighbouring New Zealand. The two most prevalent South African species *H. midae* and *H. spadicea* (Donovan 1808) and two species of commercial importance in North America *H. corrugata* (Wood 1828) and *H. fulgens* are examples of distant temperate species.

Twenty-two microsatellite primer pairs were developed for *Haliotis rubra* (Table 3-1) using methods described in Chapter 2, published as Evans *et al.* (2000). Their potential for cross-species amplification was tested under standard PCR conditions with DNA extracted from gill or muscle tissue from at least two individuals of the 12 test species using a modified CTAB protocol (Grewe *et al.* 1993). Such low sample sizes are a problem for determination of diversity indices or population structure, but are sufficient for the detection of the locus in another species.

PCR reactions were performed in a volume of 25 µL consisting of 67 mM TrisHCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 $mg.mL^{-1}$ gelatin; 2.5 mM $MgCl_2$; 10 pmoles of each primer; 200 µM dNTPs; 0.5 U *Taq* F1 polymerase (Fisher Biotech); and ~ 20 ng genomic DNA template. Amplification was in a Perkin Elmer 9600 thermocycler with one cycle of 94° C for 1 min, 50° C for 15 s and 72° C for 1 min, followed by 30 cycles of 94° C for 15 s, 50° C for 15 s and 72° C for 1 min. These cycles were followed by a final extension step of 72° C for 10 min. Amplification products were separated on 2% TBE agarose gels, and visualised under UV illumination after ethidium bromide staining. Markers were scored as present when a single band of between 75 and 450 bp was detected. Any amplification products above this size range, although possibly containing the microsatellite repeat unit, can not be scored on the ABI377 using the size standards commonly available. It is possible to score larger alleles with different systems, but such large products increase the possibility of mutation in the regions flanking the microsatellite rather than within the repeat unit. In such cases it would be best to design new primers closer to the repeat unit. Amplification products less than 75 base pairs in size are difficult to reliably score due to their proximity to such PCR artifacts as primer-dimer, and unincorporated dyes.

Table 3-1 Characterization of 22 microsatellite primer pairs tested for cross-species amplification in this study. Previously published primers appear last, and a citation is given instead of full sequence. The two primer pairs preceeded by the "* " both amplify the same locus, with *CmrHr* 2.9 being internal to *CmrHr* 2.15.

Locus	Repeat Sequence	Primer Sequence (5'-3') (F-Forward, R-Reverse)	Accession Number	Approx. Size in H.rubra(bp)
CmrHr 1.5	$(CAGA)_5$	F-GGAAGAGGTATCGTAAACTG	AF302824	126
		R-AGTCTCCCTGGTAAAACG		
CmrHr 1.6	$(CA)_4..(CA)_3$	F-GTTGTAAATGATGCCCTC	AF302825	89
		R-CGTCTTTTTATTCAACGCC		
CmrHr 1.23	$(AC)_{32}$	F-GCTGGGAAATCAATCTTC	AF302826	122
		R-CCTCACTTTCAACACTCAC		
CmrHr 2.3	$(GT)_{14}TT(TG)_3$	F-CCAGGCCCTATTCTTTCACA	AF302827	100
		R-CGTCGCACTAAACACTGCAT		
CmrHr 2.5	$(GT)_{21}$	F-GCGCAGACATTCATCGGATA	AF194955	283-299
		R-GTCCATCGTCGACAGGTTTT		
**CmrHr* 2.15	$(CA)_{27}$	F-TTTACATCGCATCGGCATTA	AF194956	288
		R-TACTTAACGTTGCCCTGCCT		
CmrHr 2.17	$(GT)_{38}$	F-AGGACTTGCCCAACCTTTTT	AF302828	226
		R-TTACAGAACAAACACAAGTATTGAA		
CmrHr 2.18	$(GAGT)_3$	F-GCTCCAGAATTCAAGGGTTG	AF302829	134
		R-GCTGCTTAACCTCAGGATGC		
CmrHr 2.20	$(AC)_{23}(GCAC)_{18}$	F-TTTTGAATGATTGTATTTCGTTTT	AF302830	186
		R-TACCTTGCATCGTAATAAACAGACAC		
CmrHr 2.22	$(CA)_{22}$	F-GGGTCGTCAGGTAGGTAGCA	AF302831	117-193
		R-CCATAATCAGAGGGGAAGCA		
CmrHr 2.23	$(AC)_{16}$	F-TGGAAGCTTTTCAAACATTGG	AF302832	258-266
		R-TACAATGGGGATTAAGAAGC		
CmrHr 2.27	$(GT)_{17}(GCGT)_{23}(GT)_2$	F-GTCCAGGTCCACAGCTCATT	AF302833	347
		R-GGAATTGAAGACCCTCCTCC		
CmrHr 2.29	$(CA)_{58}$	F-TGATTGGTGTGTGAGGTGAAA	AF302834	321
		R-CCGATGCCCTTATCATCACT		
CmrHr 1.11	$(AC)_{15}$	Chapter 2, Evans *et al.* 2000	AF194951	172-176
CmrHr 1.14	$(GT)_{13}TT(GT)_2GA\ (GT)_3$	Chapter 2, Evans *et al.* 2000	AF194952	252-262
CmrHr 1.24	$(AT)_8$	Chapter 2, Evans *et al.* 2000	AF194953	216-236
CmrHr 1.25	$(CA)_{25}(AT)_6TT(AT)_5(TG)_3$	Chapter 2, Evans *et al.* 2000	AF194954	291-309
**CmrHr* 2.9	$(GT)_{27}$	Chapter 2, Evans *et al.* 2000	AF194956	156-202
CmrHr 2.14	$(GAGT)_8..(GAGT)_5$	Chapter 2, Evans *et al.* 2000	AF194957	209-235
CmrHr 2.26a	$(ATTC)_5T_4C\ (ATTC)_2$	Chapter 2, Evans *et al.* 2000	AF194958	190-212
CmrHr 2.30	$(GT)_6..(GT)_{13}$	Chapter 2, Evans *et al.* 2000	AF194959	284-328
CmrHr 2.36	$(AC)_{21}$	Chapter 2, Evans *et al.* 2000	AF194960	83-121

3.2.2 H. midae optimisation.

The loci that produced an amplification product for *H. midae* under standard conditions were further examined to optimise PCR parameters. Samples were initially subjected to PCR amplification at temperatures of 50 and 55° C and with DNA template concentrations of

10 ng.μL^{-1} and 2 ng.μL^{-1}. Amplification products for all 10 markers were not improved by increasing the annealing temperature from 50 to 55° C, but were improved by increasing the DNA template concentration to 10 ng.μL^{-1} in all samples.

The markers were amplified in 8 individuals of *H. midae* from Cape Hangklip on the south-west coast of South Africa. Products were diluted relative to amplification strength and mixed with formamide, loading dye and Genescan Tamra500 size standard (PE-Applied Biosystems), denatured at 95° C for 2 min and 1.2 μL loaded onto a 4% denaturing polyacrylamide gel. Samples were run on an ABI377 DNA autosequencer and genotypes determined using Genotyper® software. Allele variation was scored between the 8 individuals.

3.2.2 H. laevigata and H. fulgens optimisation.

All loci that produced an amplification product in one of these two species under standard amplification conditions were subjected to further testing for optimisation. This included a range of annealing temperatures from 48° C to 58° C, "Touchdown-PCR" to improve primer specificity, where the annealing temperature at the beginning of the cycling program was high and then lowered by either 0.5 or 1.0° C each cycle until the lowest selected annealing temperature was reached. In addition, DNA template concentrations tested ranged from 1 ng.μL^{-1} to 30 ng.μL^{-1}, and $MgCl_2$ concentrations tested ranged from 1 mM to 5 mM. All loci were tested on at least 20 *H. laevigata* or 8 *H. fulgens* individuals.

3.3 Results

3.3.1 Microsatellite amplification

Nineteen of the twenty-two primer pairs tested successfully amplified a product in at least one species other than *H. rubra* (Table 3-2). Not suprisingly, the species that appears to have retained the most loci, at 15, is *H. conicopora*, a species that has been touted as perhaps a sub-species of *H. rubra* (Geiger 2000). The three other temperate Australian species (*H. laevigata, H. scalaris, H. roei*) produced an amplification product from twelve of the 22 primer pairs. *Haliotis asinina*, the only tropical species included in the study showed sequence conservation in only 5 of the markers tested. The 3 species from New Zealand showed conservation of 5, 5 and 9 markers for *H. iris, H. australis* and *H. virginea* respectively. As expected there was little cross-species amplification seen in the North American species *H. rufescens* and *H. fulgens* (3 markers each), which were shown to be in a distant clade to *H. rubra* by Lee and Vacquier (1995). It should be noted that the three loci producing an amplification product in the two North American species were the only loci to amplify a product in all 12 species tested. Interestingly though, the South African species showed more sequence conservation than the species from New Zealand, with 10 markers being conserved in *H. midae* and 9 in *H. spadiceae*. None of the primer pairs were shown to be specific to all Australian species or

to particular climatic regimes such as temperate and tropical species. Some primer pairs produced an amplification product that was dramatically different in size to that expected. Where that product was greater than 450 or less than 75 base pairs the marker was denoted by an "a" for altered product size. Although these altered products may contain the same microsatellite as other amplification products, they can be of little use if they can't be scored reliably. Further sequencing and the design of new PCR primers may prove useful however.

Table 3-2 Cross-species amplification using primers designed for *Haliotis rubra*. Assays producing a PCR product of expected size are indicated by +, those producing multiple bands or no product as -, and those producing bands of an altered size to that expected are represented by an "a"

	CmrHr	1.5	1.6	1.11	1.14	1.23	1.24	1.25	2.3	2.5	2.9	2.14	2.15	2.17	2.18	2.20	2.22	2.23	2.26	2.27	2.29	2.30	2.36	Total
Australia	*H.rubra*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	22
	H.laevigata	-	+	-	+	-	+	a	+	a	-	+	-	+	a	+	+	+	-	+	+	+	-	12
	H.scalaris	-	+	-	-	+	+	-	+	+	-	+	-	+	a	+	-	+	-	+	+	+	-	12
	H.conicopora	+	+	+	+	-	+	-	+	+	+	+	+	-	-	+	a	+	-	+	+	+	-	15
	H.roei	+	-	-	+	-	+	-	+	a	+	+	+	-	-	+	a	+	a	+	+	+	-	12
	H.asinina	-	-	-	-	+	a	-	+	a	-	-	-	-	-	+	a	-	-	+	-	+	-	5
New Zealand	*H.iris*	-	-	-	-	-	a	-	+	-	-	-	-	-	-	+	a	a	-	+	-	+	+	5
	H.australis	-	-	+	-	-	a	-	+	-	-	-	-	-	-	+	-	a	-	+	-	+	-	5
	H.virginea	-	-	-	-	-	a	-	+	+	+	+	+	-	-	+	-	+	-	+	-	+	-	9
South Africa	*H.midae*	-	a	-	-	+	+	-	a	a	+	-	+	-	-	+	-	+	-	+	+	+	+	10
	H.spadicea	+	+	-	-	+	a	-	+	+	-	-	+	-	-	+	-	-	-	+	-	-	+	9
North America	*H.fulgens*	-	-	-	-	-	-	-	+	a	a	-	-	-	-	+	a	-	-	+	-	-	-	3
	H.corrugata	-	-	-	-	-	-	-	+	a	a	-	-	-	-	+	-	-	-	+	-	-	-	3
	Total	4	5	3	4	5	6	1	12	5	5	6	6	3	1	13	2	7	1	13	6	10	4	

Previously, researchers have simply reported agarose gel detection as the retention of a locus in a related species. In this paper I attempt to bridge the gap between identifying the presence of a marker in a related species, and the use of that marker for further research. I present here, three case studies, in which I have taken the markers identified from the preliminary screening tests and attempted to optimize them for routine research in *Haliotis midae*, *H. laevigata*, and *H. fulgens*.

3.3.2 Haliotis midae-the South African abalone.

The 10 loci shown to produce an amplification product from *H. midae* in the preliminary screening were: *CmrHr* 1.23, *CmrHr* 1.24, *CmrHr 2.9*, *CmrHr* 2.15, *CmrHr* 2.20, *CmrHr* 2.23, *CmrHr* 2.27, *CmrHr* 2.29, *CmrHr* 2.30, *CmrHr* 2.36 (Table 3-2).

At the initial PCR amplification conditions, both *CmrHr* 2.27 and *CmrHr* 2.30 produced non-specific products when examined using the more sensitive automated detection techniques. These loci were re-amplified at annealing temperatures of between 50 and 58° C and with

[$MgCl_2$] of between 2.0 and 3.0 mM. The resultant amplification products however were also non-specific and these markers were not examined further.

Two of the markers were monomorphic in the 8 individuals examined and were not tested further in this study. These markers were, *CmrHr* 1.24 and *CmrHr* 2.27. It is unlikely that these markers are actually the same locus as that amplified in *H. rubra*, as they were somewhat smaller than the product produced in that species, and did not show characteristic microsatellite amplification profiles.

The variation at each of the remaining 6 loci ranged from the minimum of 2 alleles at *CmrHr* 2.30 (226 - 242 bp), 3 alleles at *CmrHr* 2.23 (244 - 252 bp), 4 alleles at *CmrHr* 2.36 (101 - 119 bp), 6 alleles at *CmrHr* 2.15 (250 - 280 bp), 6 alleles at *CmrHr* 2.9 (173 - 203 bp) and a maximum of 8 alleles at *CmrHr* 2.29 (425 - 469 bp).

3.3.3 Haliotis laevigata- The greenlip abalone

Of the 12 markers identified as being conserved in *H. laevigata* (Table 3-2), only five proved to be reliable for further studies after evaluation in 20 greenlip individuals. The reasons for exclusion of the other 7 markers are listed in Table 3-3, but were due to either non-specific or unreliable amplification. Touchdown PCR cycles failed to clean up the peak profiles of locus *CmrHr* 2.22. Two loci, *CmrHr* 1.6 and *CmrHr* 1.24 were monomorphic at 81 and 228 bp respectively in the 20 individuals examined, while the remaining three loci were variable with 7, 6 and 7 alleles detected for *CmrHr* 2.14, *CmrHr* 2.23 and *CmrHr* 2.30 respectively.

Table 3-3 Microsatellite markers initially identified as conserved in *H. laevigata*, and reasons for their exclusion from further research. Sample size for testing was n=20.

Locus	accession numbers	Repeat Sequence	Reasons for exclusion
CmrHr 1.6	AF302828	$(CA)_4..(CA)_3$	**Monomorphic in *H. laevigata***
CmrHr 1.14	AF194952	$(GT)_{13}TT(GT)_2GA(GT)_3$	Non-specific
CmrHr 1.24	AF194953	$(AT)_8$	**Monomorphic in *H. laevigata***
CmrHr 2.3	AF302827	$(GT)_{14}TT(TG)_3$	Non-specific
CmrHr 2.14	AF195957	$(GAGT)_8..(GAGT)_5$	**Suitable for research**
CmrHr 2.17	AF302828	$(GT)_{38}$	Non-Specific
CmrHr 2.20	AF302830	$(AC)_{23}(GCAC)_{18}$	Unreliable amplification
CmrHr 2.22	AF302831	$(CA)_{22}$	Unscoreable-very messy peaks
CmrHr 2.23	AF302832	$(AC)_{16}$	**Suitable for research**
CmrHr 2.27	AF302833	$(GT)_{15}$	Unreliable amplification
CmrHr 2.29	AF302834	$(AC)_{55}$	Unreliable amplification
CmrHr 2.30	AF194959	$(GT)_6..(GT)_{13}(TG)_{12}(AG)_5..(TG)_3(TG)_{16}$	**Suitable for research**

Haliotis fulgens-The blue abalone from Mexico

The preliminary screening process identified 3 markers that produced an amplification product. These markers were *CmrHr* 2.3, *CmrHr* 2.20 and *CmrHr* 2.27 (Table 3-2).

PCR amplification products were however, always non-specific when visualized on agarose gels after all optimisation conditions. Each product consisted of multiple bands within a small size range, such that allele identification was unreliable.

3.4 Discussion

The development of microsatellite markers is known to be both expensive and time consuming (Wright and Bentzen 1994). Many researchers that have produced microsatellite markers for their species have therefore examined the applicability of those markers to similar questions in related species.

The use of agarose gel detection of PCR products has been utilized for the estimation of microsatellite loci conservation across species by researchers of other taxa (White and Powell 1997; Isagi *et al.* 1999). Wirth *et al.* (1999) and Rossetto *et al.* (2000), however examined these products further by denaturing polyacrylamide gel electrophoresis (PAGE) methods which reveal allele sizes and genotypes. Others have sequenced the markers in the new species to ensure that the locus being amplified does indeed match that expected (Ezenwa *et al.* 1998).

Whilst the screening of microsatellite markers in related species by sequencing techniques is obviously the most thorough method to determine marker conservation, it is also very expensive and time consuming. In instances where large numbers of microsatellite markers are being screened across many related species for which markers are not immediately required, this process may be considered to be excessive. Likewise, the optimization of primer pairs for genotyping through either radioactive labels or fluorescence primed, automated detection techniques is also time consuming and expensive. For this reason I took the simplest approach to determining marker conservation within abalone and have then followed this by detailing efforts to optimise the markers identified in the preliminary screening for use in other species.

My research shows that a simplistic approach such as the initial screening can lead to a misleadingly high number of markers appearing to be conserved in related species. I report a 60% (6 from 10) success rate in the optimisation of *H. rubra* markers for *H. midae*, 25% (3 from 12) for *H. laevigata* and 0% (0 from 3) in *H. fulgens*. Whilst the testing of molecular markers in related species is an important component in the sharing of information, it should be noted that simply determining that a product of similar size can be amplified in another species does not suggest that marker will be useful for that species.

The three markers suitable for further studies of *H. laevigata* were *CmrHr* 2.14, *CmrHr* 2.23 and *CmrHr* 2.30. Two of these three markers have been used to determine broodstock contributors in controlled spawnings of *H. laevigata* / *H. rubra* hybrids at a commercial culture facility (Chapter 7). Locus *CmrHr*2.23 was not useful in the hybrid study however, as although it was easy to score in *H. laevigata*, a third allele of equal intensity to the first two was detected in some *H. rubra* samples, and in many of the hybrid progeny.

It should also be noted that the optimal conditions for PCR amplification will vary dramatically with different thermal cyclers. This was exemplified in the transfer of 6 *H. rubra* microsatellite loci that produced clean PCR products in *H. midae* in my research in Hobart, but required extensive re-optimisation when I used them in a genetic variation study in Cape Town, South Africa (Chapter 6). The most obvious reason for this discrepancy was the large variation in ramp times between the respective PCR machines. For this reason any attempt to transfer molecular marker technology between laboratories, and particularly between species will require additional PCR optimisation at the new site. The substitution of specified reagents with those that are cheaper or more readily available may also affect amplification (Chapter 6).

One thing that is often overlooked when testing microsatellite primers is the design of the primer sites. The failure of a particular locus to amplify in another species may not mean that the microsatellite repeat is not present in that species, but simply that one or both of the primer sites have not been conserved. As the majority of microsatellite primers are published as part of a larger sequence on the Genbank (NCBI) database, the option of primer re-design is available. In this study I have designed two pairs of primers for the *CmrHr* 2.9 clone, with the *CmrHr* 2.9 primers being internal to those of *CmrHr* 2.15. What I have seen in this case is that the external primers (*CmrHr* 2.15) were conserved in the South African species, *H. spadiceae*, while the internal primers (*CmrHr* 2.9) were not (Table 3-2). It could be argued therefore that the examination of published sequences, and if necessary the re-design of primer sites, could be a more affordable solution to marker development than the creation of a new microsatellite library.

This study shows that microsatellite loci isolated from Australian blacklip abalone, *H. rubra*, can be amplified in some related *Haliotis* species, but that the likelihood of marker conservation is reduced with increasing phylogenetic distance. Rosetto *et al.* (2000) suggest that the selection of a single species from a large genera for microsatellite locus development will result in a suite of markers for most taxa in that genus. They detail only minimal PCR optimisation for the transfer of markers between species of the *Melaleuca* genus. Scribner *et al.* (1996) provide examples of high levels of marker conservation in species ranging from whales to rodents to support their results in salmon and trout from Alaska, North America and

the UK. What I have seen here, and in the previous study of abalone microsatellites (Huang and Hanna 1998) however, is a much lower rate of marker conservation between *Haliotis* species. This finding together with the very high levels of polymorphism encountered in most abalone species (*H. midae* - Chapter 6; *H. asinina* - Selvamani *et al.* 2000; *H. rubra* - Chapter 2, published as Evans *et al.* 2000), may point towards a more rapid mutation rate of microsatellite repeats and flanking sequence in abalone than that seen in other organisms.

Research on the cross-amplification of microsatellite loci within taxa should ensure that the markers are useful within that species, and do not simply produce an amplification product. My results have clearly shown that the presence of a similar sized PCR product on agarose gels is not sufficient to report locus conservation in another species. Future studies should therefore endeavor to test those products further by radioactive or fluorescent labeling methods similar to those that would be used in larger studies. This would ensure that only markers that are likely to provide reliable genetic information would be considered by researchers commencing projects on these species.

Chapter 4 Microsatellite DNA markers for analysis of population structure in the blacklip abalone, *Haliotis rubra* around south-east Australia.

This research will be published as part of a more comprehensive analysis of *H. rubra* population structure around southern Australia that I am undertaking in collaboration with Dr. Nick Elliott and Mr. Jason Bartlett at CSIRO Marine Research in Hobart.

4.1 Introduction

The blacklip abalone, *Haliotis rubra*, is one of eight recognised species of abalone in southern Australian waters (Geiger 2000), and is distributed along the south-eastern and southern coastlines of mainland Australia and around the Tasmanian coastline. *Haliotis rubra* is a large (up to 230 mm), numerous and dioecious abalone with external fertilisation (Shepherd and Laws 1974). The species has planktonic larvae that exist in the water column for approximately seven days (Sasaki and Shepherd 1995). Despite this larval duration, evidence presented by Prince *et al.* (1987, 1988) and McShane *et al.* (1988) suggest that larval dispersal is restricted and the most successful juveniles settle within metres of their parents.

Haliotis rubra supports a commercial fishery in Tasmanian waters worth almost $100 million (1999-2000 ABARE 2000). The fishery is currently managed by a combination of restricted entry, minimum legal size limits, bag limits for recreational fishers, and a Total Allowable Catch (TAC) that is divided into transferable catch units. The current TAC (2001) of 2,800 tonnes is approximately 26.8% of the world abalone harvest, and 50.1% of the Australian abalone harvest (FAO 2001, fishery statistics-capture production 1999). Ongoing research and monitoring by the Tasmanian Department of Primary Industries, Water and Energy, in collaboration with industry partners, ensures that current management strategies are sufficient for the sustainability of the resource. The Tasmanian blacklip abalone fishery is currently managed as three fishing zones, the northern, eastern and western zones, each of which has a TAC imposed. The present fishery zones however, do not necessarily represent biologically significant divisions, but rather, separate geographic regions. A thorough examination of the genetic structure of Tasmanian blacklip abalone stocks would allow existing management practices to be modified, where required, to protect any genetically isolated stocks that may exist.

The blacklip abalone is not only an important wild fishery in southern Australia, but also the basis of an expanding aquaculture industry. The culture of abalone in Tasmania has expanded dramatically in the past ten years and is still growing. Knowledge of the genetic structure of this species in the wild provides important information to ensure that the genetic diversity in closed hatchery populations is not dramatically reduced from natural levels, and that animals are not translocated, or re-seeded into areas with different genetic identities (Chapter 7). In some Australian states, the collection of broodstock for culture is restricted to specified zones

around the hatcheries. Such zoning was introduced to prevent the transfer of "alien genotypes", although evidence to support these zones is limited (Personal Communication: Harry Gorfine, Marine and Freshwater Research Institute, Victoria; Boze Hancock, Fisheries Western Australia).

Existing knowledge of blacklip abalone population structure has been gleaned from a thorough examination of variation at allozyme loci (Brown 1991), and a more recent, but cursory examination of variation at three microsatellite, two minisatellite and six RAPD DNA markers (Huang *et al.* 2000). An early study of genetic variation within the mtDNA of blacklip abalone around Tasmania (Barrett, 1989) was hampered by technical problems, but revealed no differentiation within Tasmanian samples. The genetic information obtained, when combined with studies of larval dispersal in this species (Prince *et al.* 1987, 1988), have tended to suggest the presence of some geographic structuring in the blacklip abalone population throughout its range. In his allozyme study, that examined samples from the complete species range, Brown (1991) reports large values for gene flow between samples and a large neighborhood size, but also significant differences in allele frequencies between nearby populations. Brown offers four alternative explanations for this apparent contradiction, and supports a hypothesis of predominantly local recruitment, with the high gene flow estimate being mainly governed by large local effective population sizes, rather than a high migration component. Also raised however is the possibility of predominantly local recruitment, with the high gene flow estimate (*Nm*) resulting from homogenizing selection of allele frequencies across all local populations. Huang *et al.* (2000) examine only ten individuals from each of ten sites in southern mainland Australia compared to the mean sample size of 90.3 at 17 locations examined by Brown (1991). Huang *et al.* (2000) report significant genetic subdivision at three types of molecular markers along the coastline of Victoria and New South Wales. They also report an excess of homozygotes at all three microsatellite loci across all populations and interpret this as a result of inbreeding, due to limited larval recruitment patterns and asynchronous spawning. It should be noted however that they reported no such excess at minisatellite loci, and the allozyme study of Brown (1991) revealed a significant homozygote excess at only 8 of 171 chi-squared tests performed, with only one locus showing this excess in more than one sample.

In this study, I examine genetic variation at eight microsatellite loci in seven samples of blacklip abalone from around Tasmania, two from New South Wales and one from Victoria. I compare and contrast the results obtained here with those described by both Brown (1991) and Huang *et al.* (2000). The deviations from Hardy-Weinberg equilibrium at microsatellite loci are addressed, and the utility of each form of DNA marker for detecting population structure in blacklip abalone is assessed.

4.2 Materials and Methods

4.2.1 Sample collection

Samples of *Haliotis rubra* were collected from seven Tasmanian sites, two in New South Wales and one location in Victoria (Figure 4-1). Tasmanian samples were collected and processed by staff at the Tasmanian Aquaculture and Fisheries Institute, or collected by commercial divers and the shell and viscera were obtained from a commercial abalone processor at Margate, Tasmania. All Tasmanian abalone tissue was stored at –20° C prior to processing, which involved the dissection of gill tissue and subsequent storage at –80° C. The samples from New South Wales and Victoria were collected by staff from NSW Fisheries and the Marine and Freshwater Research Institute (MAFRI) respectively. Dissected gill and muscle tissue was preserved in 70% ethanol for transport to Tasmania. Shell length was measured at the widest part of the shell for all individuals sampled, and sex was determined by an examination of gonad colour, although where this was inconclusive, the animal was recorded as being immature.

4.2.2 DNA Extraction

Total genomic DNA was extracted using either a modified CTAB protocol as described by Grewe *et al.* (1993), or commercial Qiaquick DNA extraction kits (Qiagen). Extracted DNA was stored at –20° C.

4.2.3 Polymerase Chain Reaction

Genetic variation was examined at 8 microsatellite loci, *CmrHr* 1.14, *CmrHr* 1.24, *CmrHr* 1.25, *CmrHr* 2.9, *CmrHr* 2.14, *CmrHr* 2.26, *CmrHr* 2.30 (Evans *et al.* 2000) and *RubCA1* (Huang and Hanna 1998). PCR amplifications were performed in 96-well plates (Costar) in a total volume of 25 µL. Microsatellite loci were amplified in two separate multiplex reactions comprising 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 µL of 10X buffer (670 mM Tris-HCL pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2 $mg.mL^{-1}$ gelatin), 0.66 units *Taq* DNA polymerase, 25 ng of template DNA and the reaction made up to 25 µL with sterile milli-Q water (all reagents from Fisher Biotech). The first multiplex reaction (multiplex 1) comprised 3 pmoles of each *CmrHr* 1.14 primer, 4 pmoles of each *CmrHr* 1.24 primer, 1 pmole of each *CmrHr* 2.14 primer, 4 pmoles of each *CmrHr* 2.30 primer and 3 pmoles of each *rub*CA1 primer. The second multiplex reaction (multiplex 2) comprised 5 pmoles of each primer pair for the three loci, *CmrHr* 1.25, *CmrHr* 2.9 and *CmrHr* 2.26.

All PCRs were conducted in a Perkin-Elmer 9600 thermal cycler. Cycling conditions comprised an initial denaturation step of 3 min at 94° C and concluded with a final extension step of 10 min at 72° C. Amplification involved 10 cycles of: denaturation at 94° C for 30 s;

annealing at 60-55° C for 30 s, dropping by 0.5° C per cycle; and extension at 72° C for 60 s. This was followed by a further 25 cycles of denaturation at 94° C for 30 s; annealing at 55° C for 30 s, and extension at 72° C for 60 s.

One microlitre from each amplification was diluted in 3 μL of sterile milli-Q water, and 0.7 μL of this dilution was mixed with 2 μL formamide, 0.5 μL loading dye and 0.5 μL Genescan Tamra-500 size standard (ABI), denatured for 2 min at 95° C, and loaded onto a 4.8% denaturing polyacrylamide gel. Samples were run on an ABI-377 DNA autosequencer and genotypes determined using Genotyper® software.

4.2.4 Statistical Analysis

Genetic diversity for each locus per sample site was estimated by the number of alleles per locus and by the observed (H_o) and Hardy-Weinberg expected (H_e) heterozygosity. H_o, H_e, and tests for deviations from Hardy-Weinberg Equilibrium (HWE) within samples were estimated using GENEPOP Vers. 3.2 (Raymond and Roussett, 1995). An index of heterozygote deficiency or excess (D), where $D = [H_o-H_e] / H_e$ (Selander 1970) was also calculated from the heterozygosity estimates. Significance of departure from equilibrium levels was tested by a Markov chain procedure, with significance levels determined after 400 batches of 4000 iterations each.

Linkage disequilibrium was assessed using exact tests in GENEPOP Vers. 3.2 (Raymond and Roussett 1995). Significance of departure from equilibrium levels was tested by a Markov chain procedure, as described above.

ARLEQUIN Vers. 2.000 (Schneider *et al.* 2000) was used for an analysis of variance of allele frequencies within and among populations (AMOVA), a method based on Excoffier *et al.* (1993). ARLEQUIN also permitted multi-locus estimates of Φ_{ST}, an analogue of F_{ST}, the genetic variance component attributable to population differentiation. The AMOVA method from ARLEQUIN 2000 was used to determine F_{ST} of all samples, of the seven Tasmanian samples, and of the three samples from mainland Australian waters, as well as population pairwise F_{ST} values. Hierarchical AMOVA was used to estimate F_{CT}, the proportion of variation between Tasmanian and mainland Australian samples. Significance levels are based on 100,000 steps of a Markov chain procedure.

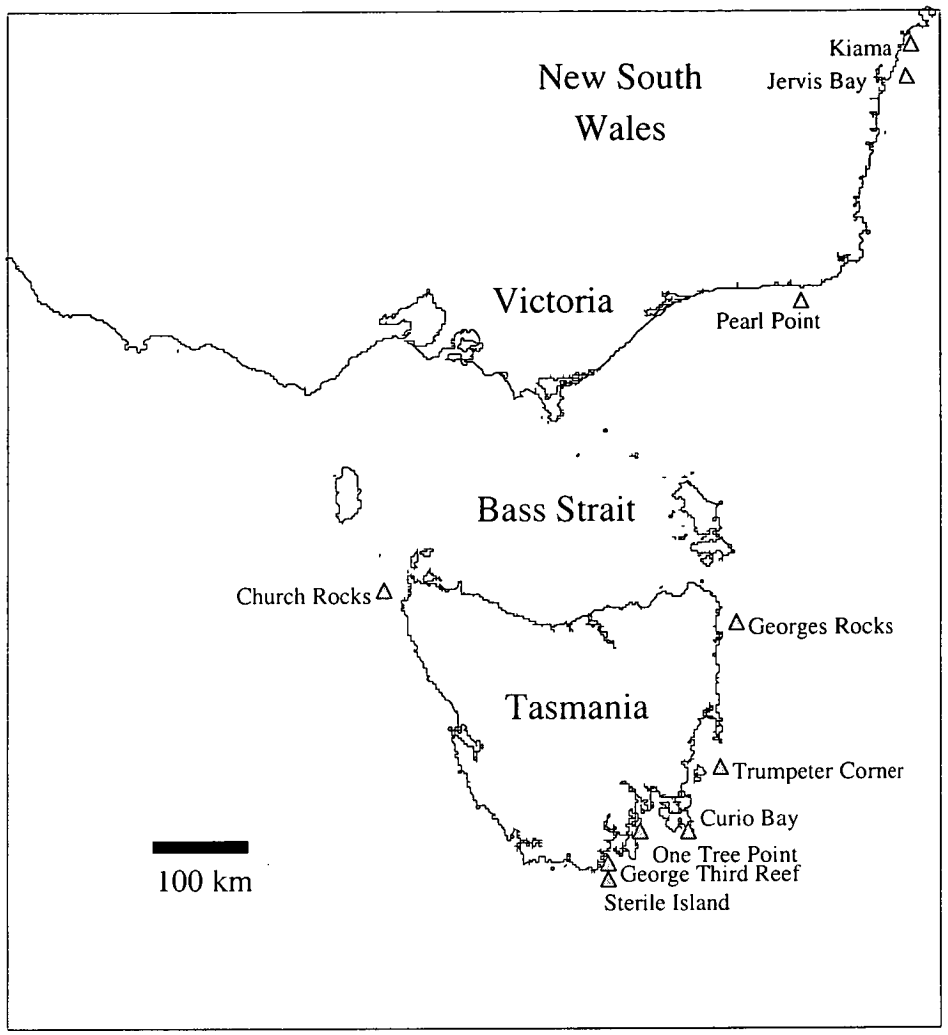

Figure 4-1 Sample sites for the collection of blacklip abalone, *Haliotis rubra* around south-east Australia.

Complete length and sex data was available for abalone from three Tasmanian samples (George Third Reef, One Tree Point, and Sterile Island). These samples were grouped and then divided into male, female and immature groups. Male and female groups were then compared using the AMOVA method of ARLEQUIN 2000 to provide an F_{ST} value. The same three samples were also divided into small (≤ 134 mm), medium (135 - 144 mm) and large (≥ 145 mm) size classes, based on shell length measured at the widest point, and another AMOVA between groups performed.

Levels of genetic differentiation among populations were also calculated using R_{ST} (as Rho). R_{ST} is an analogue of F_{ST} which has been specifically developed for the analysis of microsatellite data in that it considers such parameters as variance in allele size, relatively high

mutation rates, and utilizes a stepwise mutation model to relate alleles (Slatkin 1995). The RSTcalc package (Goodman 1997) was used to calculate Rho, an unbiased estimator of Slatkin's R_{ST} that corrects for potential biases that may result from unequal sample sizes and loci with unequal variances.

POPGENE Ver. 1.21. (Yeh *et al.* 1997) was used to produce an UPGMA dendogram, based on Nei's (1972) Genetic Distance and modified from the NEIGHBOUR procedure of PHYLIP version 3.5.

The geographic distance between all sample pairs was calculated as the shortest-by-water distance. This was done using the web based interactive mapping system developed by the Australian Surveying and Land Information Group (AUSLIG), from the Department of Industries, Science and Resources (www.auslig.gov.au). Correlation of geographic distance and genetic distance (Nei 1972) between sample pairs was tested using the correlation function of the Microsoft Excel program. Significance of correlation was determined by reference to tables of critical values of *r* in Sokal and Rohlf (1981).

4.3 Results

Genotype proportions in each population for each locus were tested for goodness-of-fit to Hardy-Weinberg expectations (Table 4-1). Forty-four of the 80 tests (8 loci, 10 samples) conducted showed significant deviation from Hardy-Weinberg expectations after sequential Bonferroni correction for multiple tests (10 samples). All significant deviations from Hardy-Weinberg equilibrium were due to an excess of homozygotes, i.e. a negative value of Selander's *D*. In this analysis, only nine tests out of the 80 conducted produced a positive value of *D* (Table 4-2). Large negative values were common, with the strongest negative response recorded at locus *CmrHr* 1.25 (mean *D* = - 0.619) revealing a large excess of homozygotes at this locus. Other loci to show consistently high negative values of *D* were *CmrHr* 2.9 (mean *D* = -0.327), *CmrHr* 2.26 (mean *D* = -0.250) and *CmrHr* 2.30 (mean *D* = - 0.188). Average *D* values for each locus across all populations were negative in all cases, indicating an overall excess of homozygotes. Over all loci, all samples showed significant departure from Hardy-Weinberg expectations. Over all samples, only locus *CmrHr* 1.24 was in Hardy-Weinberg equilibrium, and this was the locus with the least number of alleles.

Table 4-1 Genetic diversity estimates for ten Australian *Haliotis rubra* samples at eight microsatellite loci. [*N* sample size; N_{allele} number of alleles, *Mean* N_{allele} mean number of alleles per population; H_o observed heterozygosity; H_e expected heterozygosity; *P* probability of deviation from Hardy-Weinberg equilibrium.**Significant departure from Hardy-Weinberg expected equilibrium after sequential Bonferroni correction for multiple tests across loci. *All loci* provides mean values, with the exception of N_{allele} which is the sum of alleles across loci. *All loci P* value is calculated by combining probabilities across loci, and significance determined by comparison to critical values of chi-squared in Sokal and Rohlf (1981)]

Population		*CmrHr* 1.14	*CmrHr* 1.24	*CmrHr* 1.25	*CmrHr* 2.9	*CmrHr* 2.14	*CmrHr* 2.26	*CmrHr* 2.30	*rubCA*1	*All loci*
Total	*N*	*847*	*876*	*669*	*840*	*815*	*821*	*849*	*846*	*820.4*
	N_{allele}	14	8	39	41	11	16	62	43	234
	Mean N_{allele}	6.4	5.4	25.4	32.2	8.8	12.1	40.3	30.9	20.2
	Ho	0.288	0.294	0.350	0.628	0.651	0.646	0.775	0.783	0.552
	He	0.327	0.302	0.920	0.933	0.700	0.863	0.955	0.912	0.739
	P	< 0.001**	0.968	< 0.001**	< 0.001**	0.002**	< 0.001**	< 0.001**	< 0.001**	< 0.001**
Kiama	*N*	*89*	*91*	*67*	*88*	*90*	*86*	*91*	*90*	*86.5*
NSW	N_{allele}	6	5	26	36	9	12	37	28	159
	Ho	0.258	0.297	0.313	0.636	0.644	0.651	0.802	0.722	0.540
	He	0.305	0.276	0.948	0.932	0.724	0.857	0.935	0.899	0.735
	P	0.233	0.833	< 0.001**	< 0.001**	0.291	< 0.001**	0.028	< 0.001**	< 0.001**
Jervis Bay	*N*	*87*	*100*	*76*	*96*	*98*	*98*	*99*	*91*	*93.1*
NSW	N_{allele}	4	4	32	31	10	12	35	32	160
	Ho	0.172	0.280	0.289	0.583	0.643	0.684	0.798	0.582	0.504
	He	0.230	0.269	0.969	0.926	0.640	0.848	0.936	0.948	0.721
	P	0.053	1.000	< 0.001**	< 0.001**	0.743	0.037**	< 0.001**	< 0.001**	< 0.001**
Pearl Point	*N*	*80*	*89*	*55*	*88*	*85*	*79*	*87*	*82*	*80.6*
Victoria	N_{allele}	6	6	24	34	9	14	40	30	163
	Ho	0.213	0.169	0.364	0.545	0.671	0.658	0.851	0.622	0.512
	He	0.239	0.190	0.926	0.918	0.682	0.855	0.958	0.936	0.713
	P	0.049	0.351	< 0.001**	< 0.001**	0.086	< 0.001**	< 0.001**	< 0.001**	< 0.001**
Georges Rocks	*N*	*100*	*100*	*89*	*97*	*94*	*95*	*100*	*100*	*96.9*
Tasmania	N_{allele}	9	6	28	33	13	13	49	34	185
	Ho	0.360	0.350	0.382	0.557	0.585	0.537	0.860	0.790	0.553
	He	0.410	0.333	0.919	0.938	0.658	0.882	0.970	0.887	0.750
	P	0.005**	0.943	< 0.001**	< 0.001**	0.102	< 0.001**	< 0.001**	0.198	< 0.001**
Trump. Corner	*N*	*61*	*61*	*50*	*59*	*61*	*61*	*60*	*61*	*59.3*
Tasmania	N_{allele}	5	5	21	24	7	12	34	32	140
	Ho	0.328	0.377	0.360	0.559	0.623	0.623	0.850	0.918	0.580
	He	0.386	0.411	0.912	0.936	0.708	0.862	0.955	0.915	0.761
	P	0.147	0.724	< 0.001**	< 0.001**	0.575	< 0.001**	0.095	0.505	< 0.001**
Curio Bay	*N*	*92*	*91*	*66*	*92*	*81*	*72*	*87*	*92*	*84.1*
Tasmania	N_{allele}	7	5	27	34	7	12	41	31	164
	Ho	0.272	0.275	0.333	0.489	0.827	0.694	0.690	0.848	0.553
	He	0.310	0.317	0.919	0.956	0.738	0.854	0.954	0.905	0.744
	P	0.002**	0.292	< 0.001**	< 0.001**	0.022	0.001**	< 0.001**	0.114	< 0.001**

Table 4-1 Continued.

Population		*CmrHr* 1.14	*CmrHr* 1.24	*CmrHr* 1.25	*CmrHr* 2.9	*CmrHr* 2.14	*CmrHr* 2.26	*CmrHr* 2.30	*rubCA*1	*All loci*
One tree point	*N*	*91*	*90*	*78*	*87*	*72*	*90*	*89*	*86*	*85.4*
Tasmania	N_{allele}	7	7	22	34	7	13	42	33	165
	Ho	0.253	0.378	0.333	0.724	0.569	0.678	0.674	0.860	0.559
	He	0.231	0.357	0.868	0.912	0.671	0.867	0.958	0.894	0.790
	P	1.000	0.688	< 0.001**	0.001**	0.393	< 0.001**	< 0.001**	0.281	< 0.001**
George 3 Reef	*N*	*62*	*66*	*42*	*58*	*62*	*63*	*61*	*61*	*59.4*
Tasmania	N_{allele}	6	5	21	31	9	10	36	28	146
	Ho	0.323	0.197	0.357	0.776	0.597	0.635	0.672	0.787	0.543
	He	0.410	0.211	0.916	0.943	0.739	0.855	0.959	0.942	0.747
	P	0.072	0.538	< 0.001**	0.004**	0.007	0.003**	< 0.001**	0.006**	< 0.001**
Sterile Island	*N*	*90*	*92*	*64*	*83*	*80*	*82*	*79*	*87*	*82.1*
Tasmania	N_{allele}	7	6	25	31	9	11	42	31	162
	Ho	0.300	0.293	0.328	0.735	0.663	0.671	0.722	0.828	0.568
	He	0.356	0.306	0.913	0.924	0.740	0.873	0.962	0.893	0.746
	P	0.133	0.776	< 0.001**	< 0.001**	0.176	< 0.001**	< 0.001**	0.032	< 0.001**
Church Rocks	*N*	*95*	*96*	*82*	*92*	*92*	*95*	*96*	*96*	*93.0*
Tasmania	N_{allele}	7	5	28	34	8	12	47	30	171
	Ho	0.400	0.323	0.439	0.674	0.685	0.632	0.833	0.875	0.569
	He	0.392	0.352	0.912	0.946	0.695	0.874	0.964	0.897	0.754
	P	0.401	0.253	< 0.001**	< 0.001**	0.243	< 0.001**	< 0.001**	0.842	< 0.001**

Table 4-2 Selander's *D* values for ten samples of blacklip abalone at eight microsatellite loci. A negative value reveals an excess of observed homozygotes.

	Kiama	*Jervis Bay*	*Pearl point*	*Georges Rocks*	*Trump. Corner*	*Curio Bay*	*One Tree Point*	*George III Reef*	*Sterile Island*	*Church Rocks*	*Mean*
CmrHr 1.14	-0.154	-0.252	-0.113	-0.123	-0.150	-0.122	0.095	-0.213	-0.157	0.020	-0.117
CmrHr 1.24	0.074	0.041	-0.114	0.051	-0.083	-0.133	0.059	-0.067	-0.042	-0.083	-0.030
CmrHr 1.25	-0.670	-0.701	-0.607	-0.584	-0.605	-0.637	-0.616	-0.610	-0.641	-0.519	-0.619
CmrHr 2.9	-0.317	-0.370	-0.406	-0.407	-0.402	-0.488	-0.206	-0.177	-0.204	-0.288	-0.327
CmrHr 2.14	-0.110	0.005	-0.016	-0.110	-0.121	0.121	-0.152	-0.193	-0.105	-0.014	-0.069
CmrHr 2.26	-0.240	-0.193	-0.230	-0.391	-0.277	-0.187	-0.219	-0.258	-0.232	-0.277	-0.250
CmrHr 2.30	-0.142	-0.148	-0.112	-0.114	-0.110	-0.277	-0.296	-0.299	-0.250	-0.135	-0.188
rubCA1	-0.196	-0.386	-0.335	-0.110	0.004	-0.063	-0.038	-0.165	-0.073	-0.025	-0.139

The total number of alleles per locus was similar across all samples (Table 4-1). The number of alleles recorded in the samples from Trumpeter Corner and George Third Reef were slightly lower than the mean, but sample sizes at those sites were also lower. The number of individuals genotyped at locus *CmrHr* 1.25 was much lower than the mean value. This was due to amplification failure at this locus due to the presence of null homozygotes (see Chapter

5 for details). Common alleles ($P > 0.10$) were present in all samples, while unique alleles were always rare ($P < 0.02$). Complete allele frequencies are provided as Appendix A.

Linkage disequilibrium was assessed at all locus pairs in all samples and eight significant departures from equilibrium levels were detected across 280 tests ($P < 0.05$). Only one test remained significant after sequential Bonferroni correction ($P < 0.0001$). That departure occurred for the comparison of *CmrHr* 2.14 and *CmrHr* 2.9 in the Kiama sample. These two loci were not significantly linked in any other sample tested.

Overall F_{ST} values range from a low of 0.003 at *CmrHr* 2.14 to a high of 0.011 at *CmrHr* 1.25. Significant overall differentiation was observed at six of the eight loci, exceptions being *CmrHr* 1.14 and *CmrHr* 2.14 (Table 4-3), with an estimate across the eight loci and ten samples of 0.003 ($P = 0.001$). Overall R_{ST} was 0.020, suggesting highly significant ($P < 0.001$) differentiation.

Table 4-3: Genetic differentiation in *Haliotis rubra* samples at eight microsatellite loci. Estimates of overall F_{ST} and R_{ST} are presented for ten samples. [N total number of individuals; $N_{alleles}$ total number of alleles; *P* probability of significance; **Bold** *P* values significant at 0.05. **significant pairwise differentiation after sequential Bonferroni correction for multiple tests; *P* values for single locus R_{ST} was not calculated]

Locus	N	$N_{alleles}$	R_{ST}	*P*	F_{ST}	*P*
			all sites		all sites	
All	889	234	0.020	**< 0.001****	0.003	**0.001****
CmrHr 1.14	847	14	0.004	-	0.005	**0.026**
CmrHr 1.24	876	8	0.001	-	0.005	**0.015****
CmrHr 1.25	669	39	0.025	-	0.011	**0.001****
CmrHr 2.9	840	41	0.011	-	0.005	**0.002****
CmrHr 2.14	815	11	-0.002	-	0.003	0.092
CmrHr 2.26	821	16	0.008	-	0.005	**0.006****
CmrHr 2.30	849	62	0.032	-	0.010	**< 0.001****
rubCA1	846	43	0.012	-	0.004	**0.003****

Sample-pairwise F_{ST} values range from the negative value of -0.004 for the comparison of Trumpeter Corner (Maria Island, Tasmania) and Curio Bay (Tasman Peninsula, Tasmania) samples, to a maximum of 0.010 for the Jervis Bay (New South Wales) and Church Rocks (NW Tasmania) samples (Table 4-4). Fourteen sample pairs were significantly differentiated overall ($P < 0.05$), although only nine remain significant after sequential Bonferroni correction for multiple tests (45 tests). They are; Kiama and the three Tasmanian samples of Georges Rocks, Trumpeter Corner, and Church Rocks; Jervis Bay and the same three Tasmanian sites,

as well as Sterile Island. The Pearl Point sample from Victoria is significantly different to both Sterile Island and Church Rocks.

Table 4-4: Sample-pairwise differentiation as measured by overall F_{ST} at eight microsatellite loci. F_{ST} values presented below diagonal and *P* values above diagonal; **Bold** *P* values are significant at 0.05. **significant pairwise differentiation after sequential Bonferroni correction for multiple tests.

	Kiama	*Jervis Bay*	*Pearl Point*	*Georges Rocks*	*Trump. Corner*	*One Tree Point*	*Curio Bay*	*George III Reef*	*Sterile Island*	*Church Rocks*
Kiama	----------	0.396	0.775	**< 0.001**	**< 0.001**	0.676	**0.045**	0.225	0.964	**< 0.001**
Jervis Bay	0.001	----------	0.658	**< 0.001**	**< 0.001**	0.054	**0.009**	0.054	**< 0.001**	**< 0.001**
Pearl point	-0.001	0.000	----------	**0.027**	0.072	0.207	**0.027**	0.171	**< 0.001**	**< 0.001**
Georges Rocks	0.007**	0.006**	0.003	----------	0.649	0.550	0.514	0.189	0.964	0.324
Trump. Corner	0.008**	0.008**	0.004	0.001	----------	0.964	0.982	0.360	0.658	0.451
One Tree Point	0.000	0.003	0.002	0.001	-0.001	----------	0.982	0.099	0.189	0.063
Curio Bay	0.003	0.006	0.004	0.001	-0.004	-0.002	----------	0.568	0.198	0.991
George III Reef	0.002	0.004	0.003	0.003	0.001	0.003	0.000	----------	**0.036**	0.144
Sterile Island	-0.002	0.006**	0.004**	-0.002	0.000	0.002	0.002	0.004	----------	0.766
Church Rocks	0.007**	0.010**	0.007**	0.001	0.001	0.003	-0.002	0.003	-0.001	----------

Sample-pairwise values of R_{ST} averaged over variance components range from the negative value of -0.005 recorded between the Pearl Point sample in Victoria and the George Third Rock sample in southern Tasmania, to a value of 0.099 between the samples from Jervis Bay in New South Wales and Church Rocks in north-west Tasmania (Table 4-5). Eighteen sample pairs are significantly differentiated at $P < 0.05$, and 12 of these tests remain significant after sequential Bonferroni correction for multiple tests (45 tests; Table 4-5).

Table 4-5 Sample-pairwise R_{ST} values presented below diagonal. *P* values above diagonal. **Bold** *P* values significant at 0.05. **significant pairwise differentiation after sequential Bonferroni correction for multiple tests.

	Kiama	*Jervis Bay*	*Pearl Point*	*Georges Rocks*	*Trump. Corner*	*Curio Bay*	*One Tree Point*	*George III Reef*	*Sterile Island*	*Church Rocks*
Kiama	----------	0.055	0.120	**< 0.001**	**0.001**	**0.001**	0.189	0.072	0.468	**< 0.001**
Jervis Bay	0.013	----------	**0.003**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Pearl point	0.010	0.030	----------	**0.042**	0.230	0.051	0.391	0.932	0.409	**0.002**
Georges Rocks	0.043**	0.083**	0.013	----------	0.740	0.385	**0.015**	0.129	**0.012**	0.596
Trump. Corner	0.043**	0.087**	0.007	-0.003	----------	0.529	0.119	0.458	0.067	0.146
Curio Bay	0.038**	0.092**	0.013	0.002	0.000	----------	0.097	0.300	0.060	0.359
One Tree Point	0.006	0.043**	0.002	0.018	0.012	0.009	----------	0.672	0.871	0.003
George III Reef	0.015	0.045**	-0.005	0.010	0.001	0.004	-0.002	----------	0.381	**0.016**
Sterile Island	0.001	0.036**	0.002	0.018	0.015	0.013	-0.004	0.002	----------	**0.001**
Church Rocks	0.053**	0.099**	0.025	0.000	0.007	0.002	0.027	0.021	0.027**	----------

Very little of the differentiation observed was associated with differences between Tasmanian samples (Tables 4-4 and 4-5), but appeared to be due to differences between the three mainland samples and some Tasmanian samples. This was confirmed as an AMOVA derived, overall F_{ST} detected no differentiation between the seven samples in the Tasmanian group (differentiation was significant at *CmrHr* 2.30, but not overall), or between the three samples from mainland Australia (Table 4-6), but did detect significant genetic differentiation between the two groups (Tasmanian samples, and mainland samples) at each locus, and overall (F_{CT} value for differentiation between the two groups was 0.004, $P < 0.001$; Table 4-6).

Table 4-6: AMOVA derived F_{ST} values for differentiation within 7 Tasmanian samples, within 3 mainland Australian sites, and F_{CT} value for differentiation between Tasmanian and mainland samples. **Bold** *P* values are significant at 0.05. **significant differentiation after sequential Bonferroni correction for multiple tests.

Locus	F_{ST}	*P*	F_{ST}	*P*	F_{CT}	*P*
	Tas sites		mainland	sites	Tas V mainland	
All	0.001	0.602	0.000	(0.730)	0.004	**< 0.001****
CmrHr 1.14	0.003	0.145	0.003	(0.263)	0.005	**< 0.001****
CmrHr 1.24	0.002	0.183	0.003	(0.140)	0.005	**< 0.001****
CmrHr 1.25	0.007	0.111	0.008	(0.184)	0.008	**< 0.001****
CmrHr 2.9	0.001	0.647	0.000	(0.858)	0.009	**< 0.001****
CmrHr 2.14	0.002	0.257	0.004	(0.101)	0.001	**< 0.001****
CmrHr 2.26	0.006	**0.015**	0.001	(0.486)	0.002	**< 0.001****
CmrHr 2.30	0.009	**< 0.001****	0.002	(0.204)	0.006	**< 0.001****
rubCA1	0.001	0.205	0.005	(0.058)	0.003	**< 0.001****

The sex ratio of 234 mature abalone from One Tree Point, George Third Reef and Sterile Island samples was 1 female to 0.98 males, with 7.5% of animals immature. No significant allele differences at the eight loci were observed between male and female groups (F_{ST} = 0.000; $P = 0.663$), or between the three length classes examined ($F_{ST} = 0.002$; $P = 0.131$).

Nei's (1972) genetic distance measurements between pairs of samples are represented in an UPGMA dendogram (Figure 4-2). The pairwise values (not shown) range from a minimum of 0.019 (Georges Rocks and Church rocks; Kiama and Jervis Bay) to a maximum of 0.057 (Jervis Bay and Trumpeter Corner). The dendogram separates the samples from mainland Australia into a unique grouping from the Tasmanian samples. Although none of the groupings within those two branches are significant (pairwise F_{ST} values, Table 4-4), the groupings are consistent with geography. Within the mainland grouping, the two New South Wales samples are grouped together, separate to the sample from Victoria. Within the Tasmanian clade the two closest populations at the south of the island, George Third Reef and

Sterile Island are clustered together, as are the two most northern samples, Georges Rocks and Church Rocks. The eastern Tasmanian samples of Trumpeter Corner, Curio Bay and One Tree Point fall in between these two groupings.

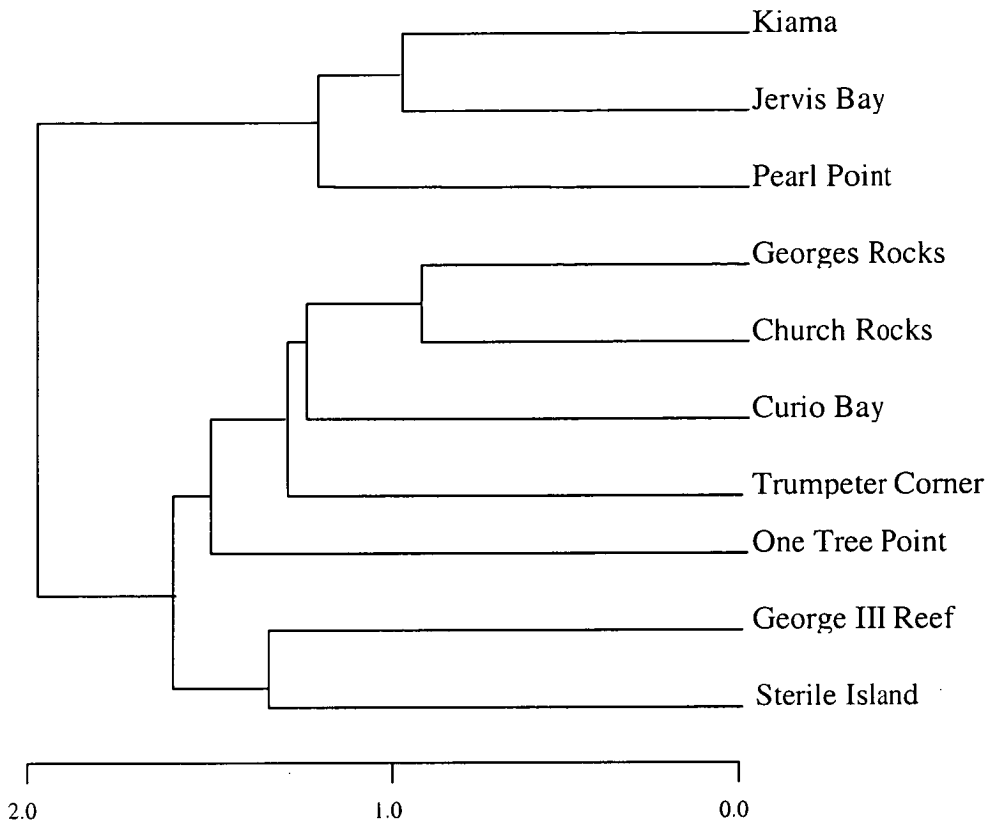

Figure 4-2 Representation of Nei's 1972 genetic distances between ten samples of blacklip abalone, *H. rubra*, showing differentiation between mainland Australian (Kiama, Jervis Bay and Pearl Point) and Tasmanian samples.

4.4 Discussion

Previous research into the genetic structure of abalone populations has yielded variable results (Table 4-7). Strong genetic structuring has been reported in *H. cracherodii* along the central Californian coast (Hamm and Burton 2000), and in *H. rubra* around southern Australia (Huang *et al.* 2000). Weaker structure was detected in studies of *H. roei, H. rubra* and *H. laevigata* (Hancock 2000; Brown and Murray 1992) populations in Australia. Studies of *H. fulgens* in Baja California and *H. rufescens* populations along the Californian coast revealed no evidence of genetic structuring (Zúñiga *et al.* 2000; Burton and Tegner 2000).

Table 4-7 Examples of genetic variation among abalone populations. Average number of individuals sampled at each location is presented as mean N. F_{ST} is the degree of population differentiation and *P* is the probability of significant departure from panmixia, as reported in each study. NS = not significant. np = not provided

Author	Species	Loci	Locations	mean N	F_{ST}	*P*
Allozymes						
Brown 1991	*H. rubra*	12	17	90.3	0.022	np
Brown & Murray 1992	*H. laevigata*	13	8	72.4	0.014	np
Burton & Tegner 2000	*H. rufescens*	4	3	45.0	0.012	NS
Hamm & Burton 2000	*H. cracherodii*	3	7	61.0	0.039	< 0.001
Hancock 2000	*H. roei*	8	10	62.4	0.009	< 0.001
Zúñiga *et al.* 2000	*H. fulgens*	7	5	20.4	0.036	NS
Microsatellites						
Huang *et al.* 2000	*H. rubra*	3	10	10.0	0.067	< 0.001
Evans (this study)	*H. rubra*	8	10	82.0	0.003	< 0.001
RAPDS						
Huang *et al.* 2000	*H. rubra*	84	10	10.0	0.074	< 0.001
Minisatellites						
Huang *et al.* 2000	*H. rubra*	2	10	10.0	0.001	NS

Brown (1991) observed average heterozygosity at twelve allozyme loci in *H. rubra* to be 0.136 (H_e = 0.143). Huang *et al.* (2000) observed values of 0.645 for two minisatellite (H_e = 0.610), and 0.313 for three microsatellite loci (H_e = 0.908). In my study an average observed heterozygosity (H_o) across eight microsatellite loci in 10 samples of *H. rubra* was 0.552, and Hardy-Weinberg expected heterozygosity was 0.739. An excess of homozygotes is apparent in both studies of *H.rubra* that have investigated variation at microsatellite loci. The homozygote excess observed by Huang *et al.* (2000) averaged across three loci was considerably larger (*D* = -0.687) than the mean value of *D* recorded at any of the loci in the present study (Table 4-2), and much higher than the mean value across 8 loci of *D* = -0.217. In the present study, locus *CmrHr* 1.25 produced the largest negative values of Selander's *D*, as well as revealing the presence of null homozygotes in individuals from all samples. In subsequent research (Chapter 5) the presence of null alleles was confirmed at this locus, and also at locus *CmrHr* 2.30 by their amplification using new primers designed in the flanking region, outside of the original primer pair. The presence of null alleles in a sample will cause an apparent homozygote excess in the data, by the incorrect genotyping of null allele containing heterozygous individuals as homozygotes for the amplifying allele.

Both the present study and that of Huang *et al.* (2000) examined genetic variation at the *rubCA1* locus (Table 4-8). Expected heterozygosity at this locus was similar in both studies (this study = 0.912; Huang *et al.* 2000 = 0.955), but observed heterozygosities varied greatly (this study = 0.783; Huang *et al.* 2000 = 0.380). Huang *et al.* (2000) considered the possibility of null alleles and the mis-typing of results contributing to the low observed heterozygosity

values in their data, but concluded that neither was likely. The large difference in observed heterozygosities between the two studies is difficult to explain in a biological sense. Huang *et al.* (2000) argue that the dramatic homozygote excess seen at all three microsatellite loci in their study is an indication of inbreeding in abalone populations. The larval recruitment research of McShane *et al.* (1988) and Prince *et al.* (1987; 1988) suggest that a large proportion of abalone larvae may be retained in the area of spawning, perhaps leading to inbreeding in some abalone populations and contributing to a general trend of excess homozygosity at most loci. It is however unlikely that such large deviations from H_e can be explained in this way. The levels of inbreeding required to produce such a large homozygote excess at microsatellite loci would also be expected to lead to an excess at other loci. Such an excess was not present at the two minisatellite markers examined by Huang *et al.* (2000), in fact they report a slight heterozygote excess across those two loci ($D = 0.06$), whilst Brown (1991) reports only a slight excess of homozygotes ($D = -0.049$) across 12 polymorphic allozyme loci. It would therefore seem more likely that the large homozygote excess reported by Huang *et al.* (2000) is due to some technical problem, such as the presence of null alleles, preferential amplification of alleles or perhaps the inability of digital analysis to detect and score weakly amplified alleles.

Table 4-8 Comparison of overall heterozygosity estimates at three microsatellite loci (Huang *et al.* 2000), and eight microsatellite loci (this study). Direct comparison of H_o and H_e values at the microsatellite locus *rubCA1* examined in common by Huang *et al.* (2000) and the present study.

	Evans (this study)	Huang *et al.* 2000
No. of microsatellite loci	8	3
Mean sample size	82	10
Overall Selander's *D*	- 0.217	- 0.687
rubCA1 only H_e	0.912	0.955
rubCA1 only H_o	0.783	0.380

In the present study, all significant departures from Hardy-Weinberg equilibrium were the result of heterozygote deficiencies, which are not uncommon in marine molluscs (Zouros and Pogson 1994). Significant deviations from expectations were seen at six of the eight loci, exceptions being *CmrHr* 1.24 and *CmrHr* 2.14. All populations showed significant departure from Hardy-Weinberg expectations at three loci (*CmrHr* 1.25, *CmrHr* 2.9 and *CmrHr* 2.26), whilst eight of the ten samples showed significant departure at *CmrHr* 2.30. The homozygote excess observed was not consistent across all loci (mean *D* range from - 0.030 at *CmrHr* 1.24 to - 0.619 at *CmrHr* 1.25), and therefore unlikely to be explained by inbreeding. The presence of some genetic structure between mainland Australian samples and those from around Tasmania may have led to an artificially large overall excess of homozygotes at some loci due

to the Wahlund effect (Richardson 1982). This effect would be negligible however, given the low values of F_{ST} recorded in my study. It is also unlikely that the Wahlund effect has contributed to the excess observed in individual samples as all collections were made over the smallest area possible. Brown (1991) performed 171 chi-squared tests for 12 allozyme loci in 17 samples and found that eight tests differed significantly ($P < 0.05$) from Hardy-Weinberg expectations. He concluded that inbreeding and the Wahlund effect are unlikely causes of these differences, but suggests the influence of assortative mating and selection are possible explanations for the few deviations measured at allozyme loci.

The microsatellite data presented here show similar levels of genetic diversity between samples, measured as either observed, or Hardy-Weinberg expected heterozygosity, or as the number of alleles per sample (Table 4-1).

The results of the R_{ST} and F_{ST} analyses provide similar results, with both revealing significant differentiation overall. R_{ST} values were however higher in all cases, and an order of magnitude larger than that for F_{ST} overall. This agrees with Slatkin (1995) who demonstrated that F_{ST} will tend to underestimate the true level of genetic differentiation when applied to microsatellite data. A single population pair within Tasmania was shown to be significantly different using R_{ST} values, this was the most southerly sample of Sterile Island and the north-western sample of Church Rocks. No significant differentiation of F_{ST} was identified between Tasmanian samples, or within mainland samples over all eight loci. Some differentiation within Tasmanian samples was identified at individual loci, the sample from George Third Reef for example, is significantly differentiated from all but one Tasmanian sample at *rubCA1* (data not shown), but the extent of differentiation is small and not significant when all loci were examined. The significant values of overall F_{ST} and R_{ST} reported in this study (Table 4-3) are due to significant differences between the three samples from mainland Australia and the samples from Tasmania.

In sample-pairwise F_{ST} test comparisons in ARLEQUIN (Ver 2.000), some significant differences were found when comparing mainland and Tasmanian samples, but none within either group (Table 4-4). This homogeneity of mainland and homogeneity of Tasmanian sites allowed the division of samples into two groups for AMOVA analysis. Such a hierarchical analysis revealed a significant F_{CT} value of 0.004 ($P < 0.001$) between the groups of samples from Tasmania and mainland Australia. The non-significant F_{ST} values of 0.001 ($P > 0.5$) within Tasmanian samples, and of 0.000 ($P > 0.5$) within mainland samples indicates that no significant differentiation of blacklip abalone stocks is evident within these regions.

The Tasmanian samples are separated from those around mainland Australia by Bass Strait, a region previously reported as a potential barrier to gene flow (Ward and Elliott 2001 and

references within). My results demonstrate restricted gene flow of *H. rubra* across Bass Strait. Population structure studies of flounder (van den Enden *et al.* 2000), and a venerid clam (Soh *et al.* 1998) also report limited gene flow across Bass Strait. The geographic separation of the Pearl Point sample in Victoria and the nearest Tasmanian sample of Georges Rock, is less than that between the two most distant Tasmanian samples of Church Rocks and Trumpeter Corner (Figure 4-1). However, unlike along the Tasmanian coast there is a lack of suitable habitat for larval settlement within the Bass Strait.

Whilst no significant differentiation was observed between the sexes, or between length classes of abalone in this study, it is important to note that the age, and therefore the growth rates of the individuals examined was not available. The age of blacklip abalone can be correlated to the number of layers of shell deposition, although this procedure is time consuming and the validity of measurements may vary between collection locations (Personal communication: Tim Karlov, Tasmanian Aquaculture and Fisheries Institute, Tasmania). The relationship between age and length will reach a plateau, resulting in each group containing individuals of similar size, but vastly different growth rates. This combination of fast and slow growing individuals within each group will mask any linkage between markers and growth rate. For a comprehensive examination of any possible linkage, markers should be used to investigate pedigreed family lines in culture or restocking programs (see Chapter 7).

The study by Brown (1991) reports a broad scale isolation by distance correlation, but with some geographically proximate samples being relatively genetically discrete. Brown (1991) suggests that the most plausible explanation for this data is a climate of predominantly local recruitment, with high gene flow estimates governed by large local effective population sizes, rather than high migration. The current study differs from those of Brown (1991) and Huang *et al.* (2000) because although some structure was detected between mainland Australian samples and Tasmanian samples, the Tasmanian blacklip abalone population does not fit an isolation by distance hypothesis ($r = 0.590$, $P > 0.05$, d.f. = 8), nor does it show the dramatic and consistent differentiation between samples more than 84 Km apart. My microsatellite data indicate that there is significant differentiation between some Tasmanian samples and some sites in mainland Australia, but this differentiation is not consistent across loci, or significantly correlated with geographic distance.

Population structure at any location will be influenced by the influx of a small percentage of foreign genotypes at irregular intervals. Studies of larval recruitment in abalone have revealed that a high proportion of larvae are retained within the immediate vicinity of spawning (McShane *et al.* 1988; Prince *et al.* 1987, 1988). Those that are not retained in the immediate vicinity may not encounter suitable habitat for settlement within their larval period and will therefore be lost to the system. However, on irregular occasions when oceanographic

conditions are conducive to large scale larval transport and survival, the genetic structure of any freshly colonised area may be strongly influenced by the establishment and subsequent gamete contribution of those individuals. Such conditions in concert with a large effective population size of *H. rubra* in Tasmanian waters could be used to explain these results. Any minor differentiation detected may therefore be indicative of temporal variation, a proposal that is being investigated in the expanded study by CSIRO marine Research and FRDC.

Unlike the data presented by Huang *et al.* (2000), my research reveals no overall significant difference between the samples collected in New South Wales and Victoria, despite these collections occurring over 390 Km of coastline. The marker *rubCA1*, reveals no differentiation between the Victorian sample and either of the samples from New South Wales in my study. Whilst the two New South Wales samples are differentiated from each other at this locus, the differentiation is small, and no significant differentiation is seen at other loci. This locus was used in the study by Huang *et al.* (2000) that found significant differentiation between abalone populations within the management zones of Victoria. The two studies cannot be directly compared, however, as I examined only one Victorian sample, and two from New South Wales, compared to the nine Victorian samples and one from New South Wales examined by Huang *et al.* (2000). Interestingly though, whilst the previous study identified significant differentiation between the Point Hicks sample in Victoria and the sample from Eden, 121 km away in southern New South Wales, my study showed no significant differentiation between the Pearl Point sample, approximately 40 km west of Point Hicks, and the samples from Kiama or Jervis Bay, more than 230 km north of Eden. The results presented here therefore question the findings of the earlier study, and I would reiterate that the presence of technical problems in the data collection methods of Huang *et al.* (2000) seem more likely to explain the large homozygote excess they observed, than the inbreeding of abalone populations that they suggest. The presence of such a high proportion of homozygotes in a data set, if not a real phenomenon, would exaggerate the significance of apparent differentiation detected between small samples, such as those examined by Huang *et al.* (2000).

The utility of various molecular markers to identify population structure in blacklip abalone populations around Australia has been considered previously by Brown (1991) and by Huang *et al.* (2000). Some species have been reported to have no genetic structuring at allozyme loci, while subsequent analyses using mitochondrial DNA or microsatellite markers have revealed the presence of a genetically structured population (eg. Sweijd 1999). One explanation for these differences lies in the lower rate of evolution at protein coding loci such as allozymes when compared to the non-coding microsatellite loci. The protein coding nature of allozymes has also led to their neutrality being questioned by some authors (eg. Karl and Avise 1992). In the rush to embrace the new, more powerful markers we must remember that the markers are only more powerful if they are used on statistically valid sample sizes, and high quality data

sets. A wealth of literature is available to support the need for larger sample sizes in population genetic research (see Ruzzante 1998); many of these studies call for sample sizes larger than the mean of 82 individuals per sample used in my study, but the consensus seems to require a minimum of 50 individuals in each sample (this depends on the number of alleles, and their frequencies). It is not therefore appropriate to pass judgement on the utility of markers such as RAPDs and minisatellites for population genetic research without first conducting a study of suitable magnitude. Likewise, where large variations from expected genotype frequencies are observed, our first efforts must be to affirm the quality of our data set. My analysis of eight microsatellite loci in this study shows that these markers are a useful tool for the detection of population structure in Australian blacklip abalone populations. The high levels of polymorphism reported at some loci however, may result in reduced power for detection of differentiation on a population scale. The continued development of microsatellite markers that provide an intermediate number of alleles (eg. 20 to 30 alleles) may result in more fine scale resolution of population structure in this species. The study also suggests that the high levels of homozygosity observed at some microsatellite markers may not be the result of biological interactions, but rather the influence of technical problems such as the presence of null alleles (see Chapter 5 for full explanation).

In conclusion, the data generated by the three studies of molecular diversity in the Australian *H. rubra* population agree that some structure does exist on a broad scale, detectable by allozymes (Brown 1991), microsatellites (Huang *et al.* 2000; This study) and RAPDs (Huang *et al.* 2000), but the presence of fine scale structure within the Tasmanian population and within the south-eastern mainland population remains unclear. This may be due to a lack of structure in these areas caused by infrequent and unpredictable larval transport and settlement events, or it may be due to a lack of sensitivity of the markers. A continuation of the microsatellite research to include samples covering the complete species range from eastern Victoria to western Australia is planned to confirm the broad scale differentiation between samples from Tasmania and those from mainland Australia.

The suggested presence of a single panmictic stock of abalone in Tasmania has implications to the management of the fishery. The fishery is presently managed as blocks around the state, allowing for protection of discrete populations should they exist. This study has failed to identify genetically discrete populations of abalone around the Tasmanian coast. These findings could be used to suggest that the fishery be managed as a single unit. Such a suggestion however should be treated with caution, for whilst the presence of population structure can be proved, the failure to identify structure does not prove that none exists. I would therefore recommend that the existing management methods are retained as a conservative approach to the protection of the fishery, as this is favourable to another collapse.

Chapter 5 Identification and explanation of null (non-amplifying) alleles at two *H. rubra* microsatellite loci.

5.1 Introduction

Microsatellite loci have been described as ideal markers for the measurement of population structure thanks primarily to their high polymorphism, co-dominance and abundance throughout the genome of most organisms (eg. Bowcock *et al.* 1994; Lanzaro *et al.* 1995). The high polymorphism of microsatellites results from high mutation rates, estimated to range from 10^{-2} to 10^{-5} locus.gamete^{-1} in each generation (Dallas 1992; Weber and Wong 1993).

The term "null allele" is used to describe those alleles that cannot be visualised on the gel media due to the presence of insufficient PCR product (Lehmann *et al.* 1996). It is generally accepted that null alleles are the result of a mutation(s) in the flanking sequence that is complementary to one of the oligonucleotide primers. A null allele can arise from point mutation(s) in one or both of the priming sites (Eggleston-Stott *et al.* 1997), from deletion events within the primer site(s) (Donini *et al.* 1998), or due to a large insertion between the microsatellite sequence and the primer binding site(s) (Uzunova and Ecke 1999).

Null alleles represent a common complication in the interpretation of microsatellite genotype data, resulting in a reduced level of heterozygosity (Callen *et al.* 1993). Their repeated, unintentional discovery attests to their relative abundance. Numerous studies have indicated the presence of null alleles (Ede and Crawford 1995; Eggleston-Stott *et al.* 1997; McGoldrick *et al.* 2000), although most have not attempted to identify the cause of amplification failure (eg. McGoldrick *et al.* 2000).

A consistent excess of homozyotes was recorded at eight microsatellite loci during my investigation of the population structure of the blacklip abalone, *Haliotis rubra* in south-eastern Australia (Chapter 4). Both *CmrHr* 2.30 and *CmrHr* 1.25 revealed large homozygote excess, and *CmrHr* 1.25 also resulted in failed PCRs at a low, but appreciable frequency. Amplification failure was not consistent for the same individual at other loci when amplified in a multiplex or single locus reaction, therefore ruling out the presence of non-specific inhibitors to PCR, and poor template quality. In this study I describe my attempts to firstly identify the presence of null alleles at two *H. rubra* microsatellite loci, and to subsequently identify their cause.

5.2 Identification of null alleles

Two of the loci that differed significantly from H_e in Chapter 4 were examined for the presence of null alleles, they were: *CmrHr* 1.25 and *CmrHr* 2.30. Suspected null homozygotes were common at *CmrHr* 1.25, and whilst null homozygotes were not observed at *CmrHr* 2.30, the locus consistently produced a large homozygote excess, which can be a sign of null allele presence.

New oligonucleotide primers were designed in flanking sequence outside of the original microsatellite primer pairs using OLIGO (ver. 5.0) primer design software. The new primers, named *Null* 1.25-F (5' CGG TCC TTG CCA ACA GAC TTA 3') and *Null* 1.25-R (5' TCT CAC TTC TCA ACG TTA TGG GTA A 3') were designed to amplify a product of approximately 440 bp (Figure 5-1). The new primers, named *Null* 2.30-F (5' TAA TGT GTT TGT TTT TTG GGT G 3') and *Null* 2.30-R (5' CTC TGA ACT CGA AAT CCT TAC GTA A 3') were designed to amplify a product of approximately 500 bp (Figure 5-2).

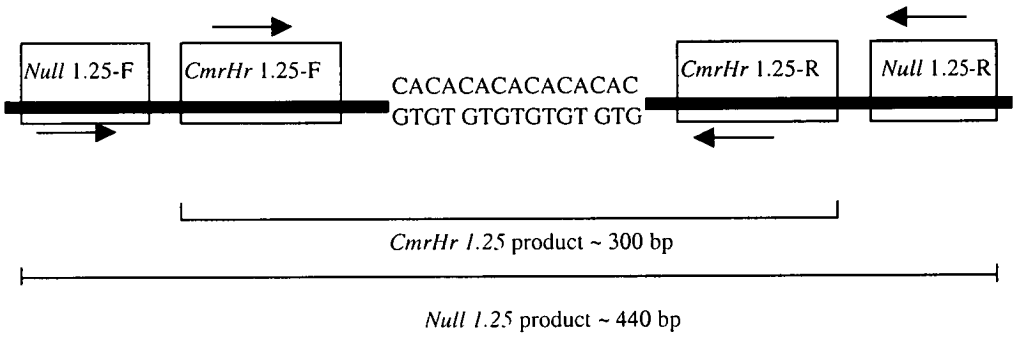

Figure 5-1 Diagramatic representation of oligonucleotide primer positions for identification of null alleles in *Haliotis rubra* at locus *CmrHr* 1.25. Arrows show 5' to 3' direction.

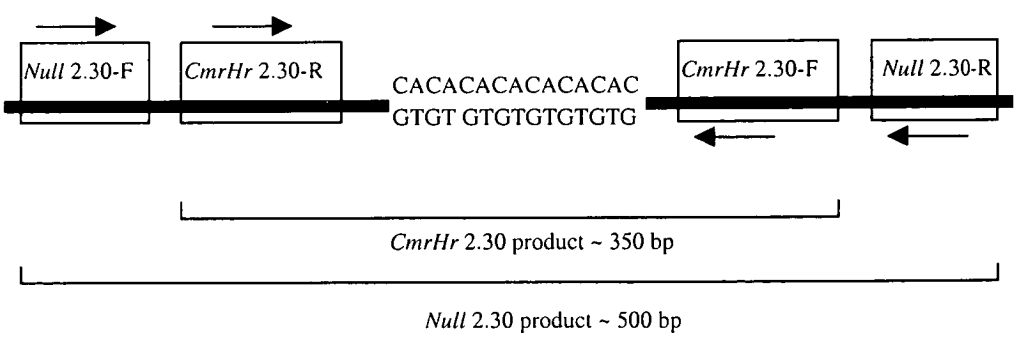

Figure 5-2 Diagramatic representation of oligonucleotide primer positions for identification of null alleles in *Haliotis rubra* at locus *CmrHr* 2.30. Arrows show 5' to 3' direction.

5.2.1 Null 1.25 samples

Extracted DNA from six individuals (labelled Null 1-6) that failed to amplify a product using the original *CmrHr* 1.25 primer pair (presumed null homozygotes), and four individuals (labelled Homo 1-4) that amplified a single allele (318, 360, 310, 302 bp) using the original primers (possible null heterozygotes) was used as template for PCRs with the *Null* 1.25 primer pair.

5.2.2 Null 2.30 samples

Extracted DNA from four individuals (labelled A-D) that produced a single allele (297, 309, 303, 329 bp) using the original *CmrHr* 2.30 primers was used as template for PCRs with the *Null* 2.30 primer pair.

5.3 PCR amplification and sequencing

All individuals were subjected to single locus PCR amplification under the conditions described in Chapter 4, with 5 pmoles of each primer used in a 25 µL reaction volume. Where amplification was successful as determined by gel electrophoresis, the resulting products were purified using Wizard spin columns (*Promega*) and cloned using the TOPO TA cloning® kit (*Invitrogen*). Eight positive colonies were then picked from a plate representing each individual, and the DNA extracted using the "rapid boil" protocol of Hamilton *et al.* (1999). Cloned alleles were retrieved by PCR amplification with T3 and T7 primers as described in the TOPO TA cloning kit guide. Allele size was determined by gel electrophoresis, and comparison to a commercial size standard (*Promega* 100 bp ladder). Products from the expected size range were purified using Wizard spin columns (*Promega*) and then sequenced in both directions using Big Dye terminator reactions (*Perkin-Elmer*), and visualized on an ABI 377 DNA autosequencer.

5.3.1 Null 1.25 long PCR

PCR of the six presumed null homozygotes at *CmrHr* 1.25 failed to produce an amplification product using the *Null* 1.25 primers under the conditions described. To test for the possibility of an insertion between the primer binding site(s) and the microsatellite sequence, a combination of *Taq* DNA polymerase and *Pfu* polymerase was used in a long PCR protocol designed to amplify products of up to 4 Kb in size (modified from Barnes *et al.* 1994). This protocol uses a 2- step PCR cycle (45 cycles of: 94° C denaturation 15 s, 68° C annealing / extension 4 min). *Pfu* polymerase (*Promega*) has a proofreading capability which enables the accurate amplification of longer DNA fragments. Loci were amplified in separate 50 µL PCR reactions comprising 250 mM Tris pH 9.1, 16 mM $(NH_4)_2SO_4$, 250 mM dNTPs, 3.5 mM

$MgCl_2$, 100 ng of each primer, 2 units *Taq / Pfu* DNA polymerase mix (80:1) and approximately 100 ng of template DNA. Reactions were made up to 50 µL with sterile milli-Q water (reagents from Fisher Biotech, unless otherwise stated). Amplification was achieved for all individuals using this protocol, and products were purified and sequenced as described above.

Sequence data was aligned with the clone sequence that the original *CmrHr* 1.25 primers were designed from using Sequence Navigator, and variation within the original primer sites and flanking region was assessed.

5.4 Results

5.4.1 Null 1.25

All homozygotes and suspected null homozygotes at locus *CmrHr* 1.25 amplified a single allele (430 - 490 bp) using the *Null* primer pair and the two-step, long-PCR process (Table 5-1). The sequence obtained for the 10 individuals examined revealed no mutation within either of the original *CmrHr* 1.25 primer binding sites in any individual.

Table 5-1 Genotypes of ten individuals at original and *Null 1.25* primers, and four individuals at original and *Null 2.30* primers. All new genotype sizes are estimated from electrophoresis on 2% agarose gels. Previously non-amplified alleles are in bold.

Individual	Original genotype	New genotype
	CmrHr 1.25	*Null* 1.25
null 1	no amp	**450/450**
null 2	no amp	**470/470**
null 3	no amp	**450/450**
null 4	no amp	**450/450**
null 5	no amp	**450/450**
null 6	no amp	**450/450**
homo 1	318/318	450/450
homo 2	360/360	490/490
homo 3	310/310	440/440
homo 4	302/302	430/430
	CmrHr 2.30	*Null* 2.30
A	297/297	466/466
B	309/309	478/**470**
C	303/303	472/**430**
D	329/329	498/**450**

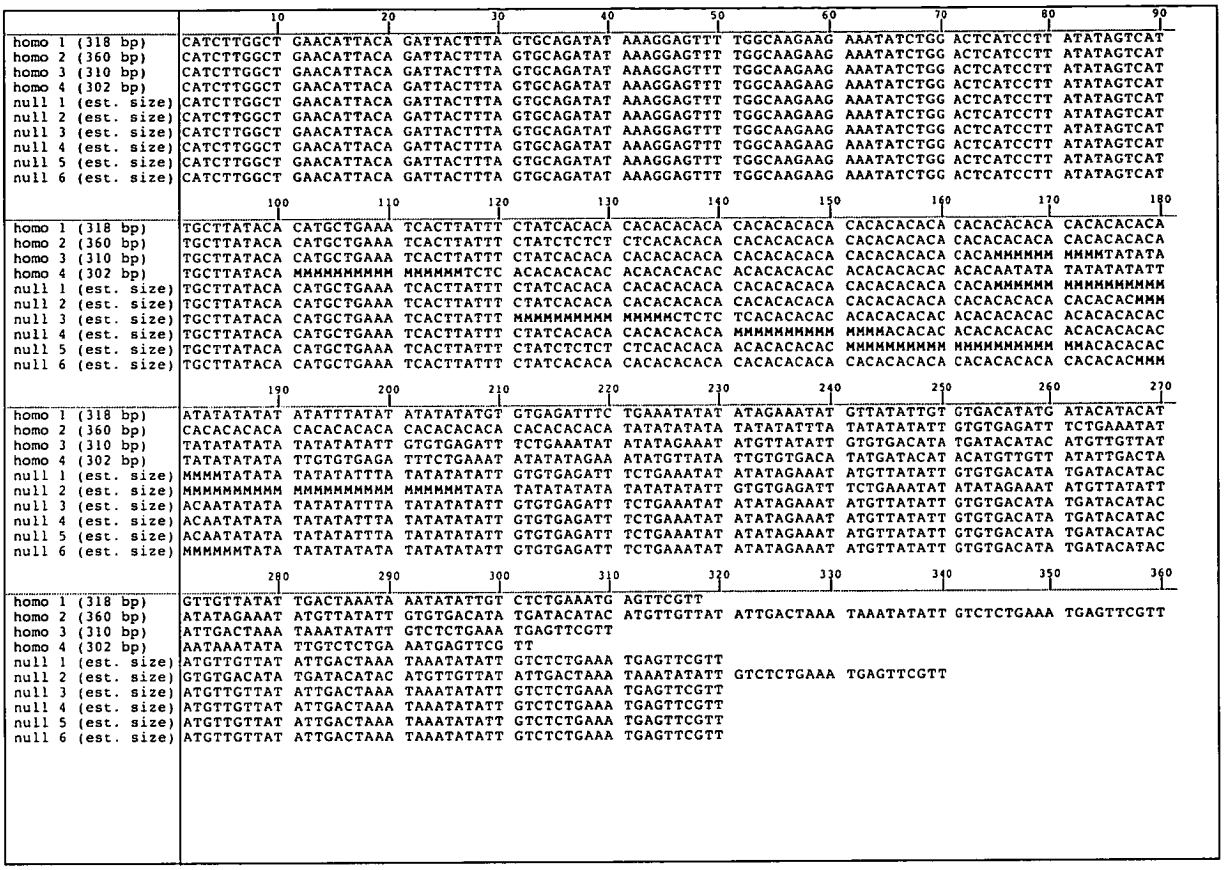

Figure 5-3 Sequence data for 6 null homozygotes (null 1-6), and 4 homozygous (homo 1-4) blacklip abalone individuals for locus *CmrHr* 1.25. Amplification with the external primer pair *Null 1.25* amplified a single allele in all individuals tested. Allele sizes for null hmozoygotes were estimated from agarose gels, allele size for homozygotes was measured using Genotyper software. M = missing data.

Some sequence differences were identified between samples therefore removing the possibility of PCR contamination, but no consistent difference was identified between amplifying and non-amplifying alleles (Figure 5-3). Allele sizes were estimated from agarose gels, as sequence overlap within the microsatellite sequence could not be confirmed.

5.4.2 Null 2.30

One individual (A) amplified a single allele for *Null* 2.30 at the size expected (466 bp; 297 bp from *CmrHr* 2.30 primer pair, plus 169 bp additional flanking region). The sequence of this individual revealed no change in nucleotide sequence within either of the original *CmrHr* 2.30 primer binding sites.

Three individuals (B, C, D) amplified a second, smaller allele in addition to the expected allele (Table 5-1). All sequence data were aligned to the original clone sequence using Sequence Navigator software (Figure 5-4).

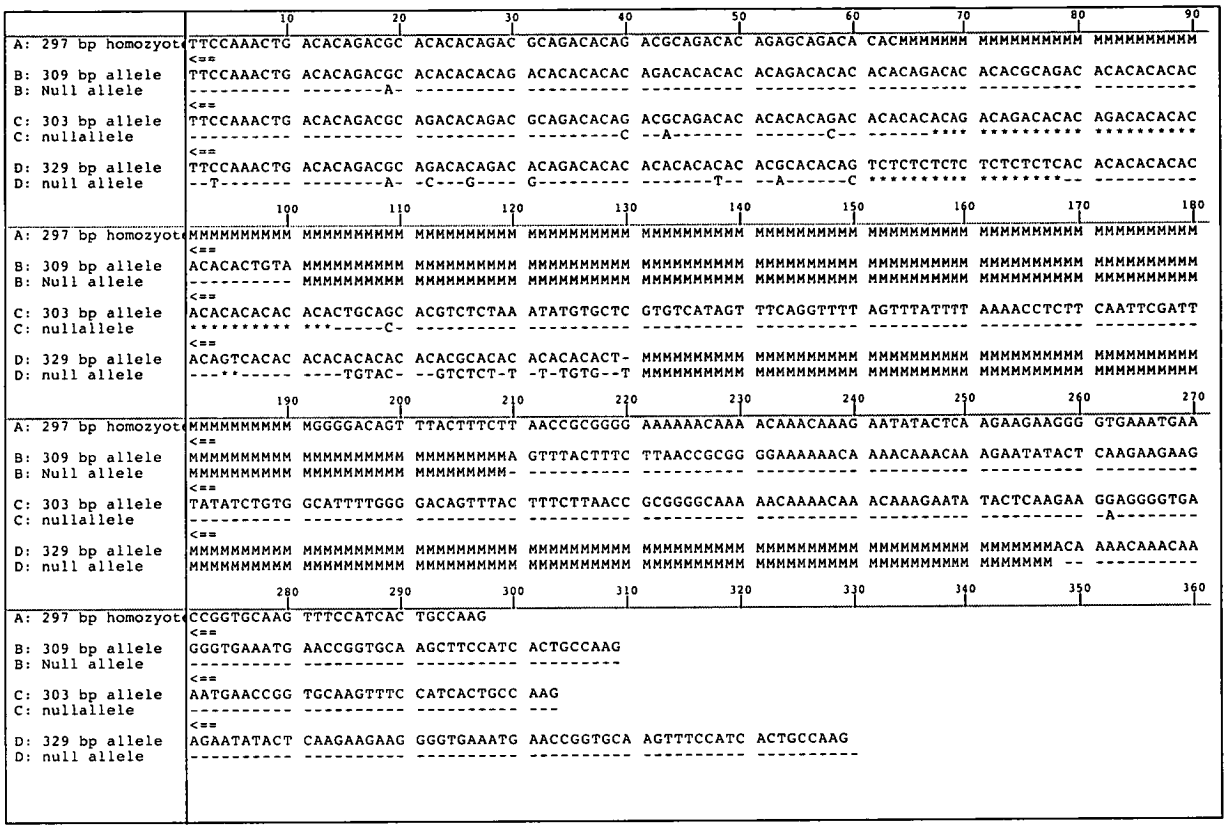

Figure 5-4 Sequence data for 4 blacklip abalone individuals that were homozygous for locus *CmrHr* 2.30. Amplification with the external primer pair *Null* 2.30 revealed a second allele in three cases. - = Shared nucleotide, * = Deletion of nucleotide, M = missing data. Size of null alleles estimated from agarose gels.

Allele sizes were again estimated from agarose gels, as sequence overlap within the microsatellite sequence could not be confirmed. Sequencing of individuals B and D revealed the presence of a G to A transversion at position 19 of the 20 mer forward primer binding site in the null allele. A second mutation (C to T transversion) was also revealed at position 3 of the same primer site in individual D.

No mutation was detected in either primer binding site in either of the two alleles sequenced for individual C. The alleles sequenced for this individual differ in size by approximately 40 bp, and it is the smaller allele (~ 430 bp) that failed to amplify (null allele) using the *CmrHr* 2.30 primer pair (Table 5-1).

5.5 Discussion

Previous studies of population structure in molluscs have ruled out the presence of null, or non-amplifying alleles as a cause of homozygote excess at microsatellite loci due to a lack of evidence in the form of null homozygotes (eg. McGoldrick *et al.* 2000). Huang *et al.* (2000) designed new primers for one of their microstellite loci that had revealed a large homozygote excess in *H. rubra* samples (not stated if these were internal or external to original primers). Individuals that appeared homozygous with the original primers, remained homozygous with the new primers, and they therefore concluded that null alleles were unlikely to be the cause of the large homozygote excess reported in their study. In my study, suspected null homozygotes were observed in all populations at the *CmrHr* 1.25 locus. Null alleles were then identified at locus *CmrHr* 1.25 and *CmrHr* 2.30 by re-amplification of these loci with new primer pairs designed outside the original primers, and sequencing through the original primer binding sites.

Three of four individuals that were homozygous for the original *CmrHr* 2.30 primer pair proved to be heterozygous when amplified and sequenced with the new, external primers. If this is representative of all samples examined then the null allele(s) at this locus may explain the large homozygote excess reported.

Four individuals that were homozygous for the original *CmrHr* 1.25 primer pair remained homozygous when amplified and sequenced with the new external primers. Six more individuals that produced no product from the original *CmrHr* 1.25 primer pair (null homozygotes), produced a single allele when amplified and sequenced with the new, external primers. The consistently large homozygote excess at this locus must also therefore, be explained, at least in part by the presence of null alelles.

Two more loci produced consistently large homozygote excesses (*CmrHr* 2.9 and *CmrHr* 2.26) in my study of *H. rubra* population structure (Chapter 4), which upon further examination may also be revealed to have null alleles. Based on this evidence, it is likely that a common cause of homozygote excess in microsatellite studies of abalone is the presence of null or non-amplifying alleles at medium to high frequency.

It should be noted here however that the cause of the non-amplification could not be determined in all cases. Null alleles are often presumed to be due to a point mutation or base insertion/deletion near the 3' end of one or both of the oligonucleotide primer sites. In some research this assumption has been supported by the use of external primers to sequence through the original primer sites in "null individuals" revealing the mutation in the original primer-binding site (Ede and Crawford 1995; Eggleston-Stott *et al.* 1997).

In this study I have used similar methods to reveal the presence of null alleles at two abalone microsatellite loci. Subsequent sequencing of null alleles has revealed the presence of point mutation(s) near the 3' end of the *CmrHr* 2.30-R primer in two individuals. However, no mutation was identified within either of the original primer binding sites for the null allele (~ 50bp smaller) in individual C. One possible explanation for the amplification failure of this allele is preferential amplification, whereby PCR conditions favour the amplification of one allele over the other. In this case it appears that the larger allele may be preferentially amplified, with the smaller allele not being amplified to sufficient quantity for detection on the gel.

Likewise, both original primer binding sites were conserved at *CmrHr* 1.25 in all six null homozygotes examined. No conclusive reason was found for this amplification failure. Poor DNA template quality would be expected to affect other loci, which was not seen here. Amplification conditions for each of the loci remained constant due to their amplification in multiplex reactions. Each of these individuals was also included in single locus reactions with both original and *Null* primers under less stringent amplification conditions with no resulting amplification. Only through the use of conditions normally reserved for the amplification of very long products was any amplification from null homozygote individuals achieved. The null alleles at this locus range in size from approximately 310 to 330 bp, all within the 290 to 368 bp allele range of this locus in natural populations (Appendix A). It is therefore unlikely that the amplification failure is related to microsatellite length. Although I was unable to obtain complete sequence for each allele due to the difficulties associated with sequencing through microsatellite repeats, the sequence obtained was aligned to the original clone sequence for this locus and searched for the presence of consistent differences between amplifying and non amplifying alleles, none of which were identified.

In conclusion, null alleles were identified at two abalone microsatellite loci. They appear to occur at relatively high frequencies in natural populations leading to a large excess of homozygotes being observed in genetic variation studies (Chapter 4). Although null alleles are generally believed to be due to mutations within microsatellite primer binding site(s), there may also be other, as yet unqualified factors, involved in the suppression of allelic amplification in some individuals. Therefore the re-design of oligonucleotide primers at null containing loci may not always result in the successful amplification of all alleles.

Chapter 6 A marked genetic discontinuity in stocks of the South African abalone, *Haliotis midae* separated by Cape Agulhas: Evidence from allozymes, mitochondrial DNA and microsatellites.

To be published as:

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6.1 Introduction

The abalone *Haliotis midae* Linn. is a highly valued marine resource. It is one of six extant species of abalone that occur on the South African coast (Muller 1986), and the only one that supports a commercial fishery (~650 tonnes.yr^{-1} since the 1970s). The management of the *H. midae* resource has been based on effort limitation and a total allowable catch (TAC) within seven fishing zones (A - G) along the western and south-western Cape coast (Figure 6-1; Tarr 1992). Minimum legal size limits, fishing seasons, bag limits for recreational divers, gear restrictions and closed areas for abalone harvest comprise the regulations that are currently part of the management strategy.

In more recent times there has been increased pressure on the management of this fishery, from both recreational fishers (Payne and Mathews 1995) and from previously excluded fishers who demand access to an already saturated fishery. Since 1995, the TAC has steadily been lowered to compensate for declining stocks. The decline has been caused primarily by illegal exploitation, which includes the over harvesting of the resource by licensed fishers, and the continual removal of undersized abalone and of individuals from within marine protected areas. Sweijd (1999) discusses the unenviable position of fishery managers in South Africa, who must consider the political and commercial demands of the community despite their obvious conflict with the biological reality of the local resource.

In an area that is under heavy fishing pressure, such as the Cape coast of South Africa, it is important that the recruitment patterns and population structure of commercial species, and interactions with other species, are fully understood. This information can determine the ability of an area of reef to recover from over exploitation, or in some cases, the complete loss of the resource. By explaining the genetic structure of the *H.midae* resource around the Cape coast, there is hope that the fishery can be managed at all levels to ensure the conservation and perhaps eventual recovery of the resource.

There has been two previous examinations of population structure in *Haliotis midae* using two genetic marker systems (Sweijd 1999). The first utilised allozyme electrophoresis, and examined variation at seven protein coding loci in five samples of *Haliotis midae*

representative of its distribution (Figure 6-1). A consistently high level of genetic variation was detected across all populations. There was little evidence of any population differentiation. Sweijd concluded that an examination of *H.midae* population structure with alternative markers was warranted. This statement is supported by examples of previous studies in which contradictory evidence has been provided by alternative genetic markers (Avise 1994; Hare and Avise 1996). In population studies of abalone, variation at allozyme loci has so far been the predominant method for examination. These have revealed strong genetic structuring in some species and little or no sub-structure in others (see Withler 2000 for a review). The second study of *H. midae* examined mitochondrial DNA variation. Sweijd (1999) used PCR to amplify two fragments of the mtDNA genome in abalone from 16 localities (Figure 6-1), representing the entire distribution of the species on the South African coast. The first fragment was approximately 1600 base pairs (bp) in length and contained part of the NADH subunit1, some tRNAs, part of the 16S rRNA gene and approximately 200 bp of unidentified sequence. The second fragment was approximately 1700 bp long and contained all of the cytochrome oxidase 3 gene and most of the NADH subunit 3 as well as a further 200 bp of unidentified sequence.

These two fragments were assayed for variation using eight restriction enzymes and analysis revealed a very clear difference in diversity measures at all localities in the two major regions, east and west of Cape Agulhas. It also showed a much higher genetic diversity in the western region; the haplotype diversity of 0.89 measured at Sea Point in the west is described as "of the highest measured in molluscs" (Sweijd 1999).

Whilst it would appear that mtDNA is an informative marker for detecting population differentiation in *H.midae*, examination with a more variable and faster evolving marker such as microsatellites may resolve more fine scale population structure. The present study examines samples from across the same range covered in the allozyme and mtDNA studies. The deployment of three independent genetic methods provides an opportunity to assess each of these techniques for their strengths and weaknesses, as well as providing a thorough examination of the genetic structuring of *H.midae* in South Africa.

6.2 Materials and methods

6.2.1 Sample collection

SCUBA divers collected commercially sized *H.midae* individuals from six localities on the South African coast (Figure 6-1), three to the west and three to the east of the genetic discontinuity described by mtDNA results. These localities were, from west to east, Dassen

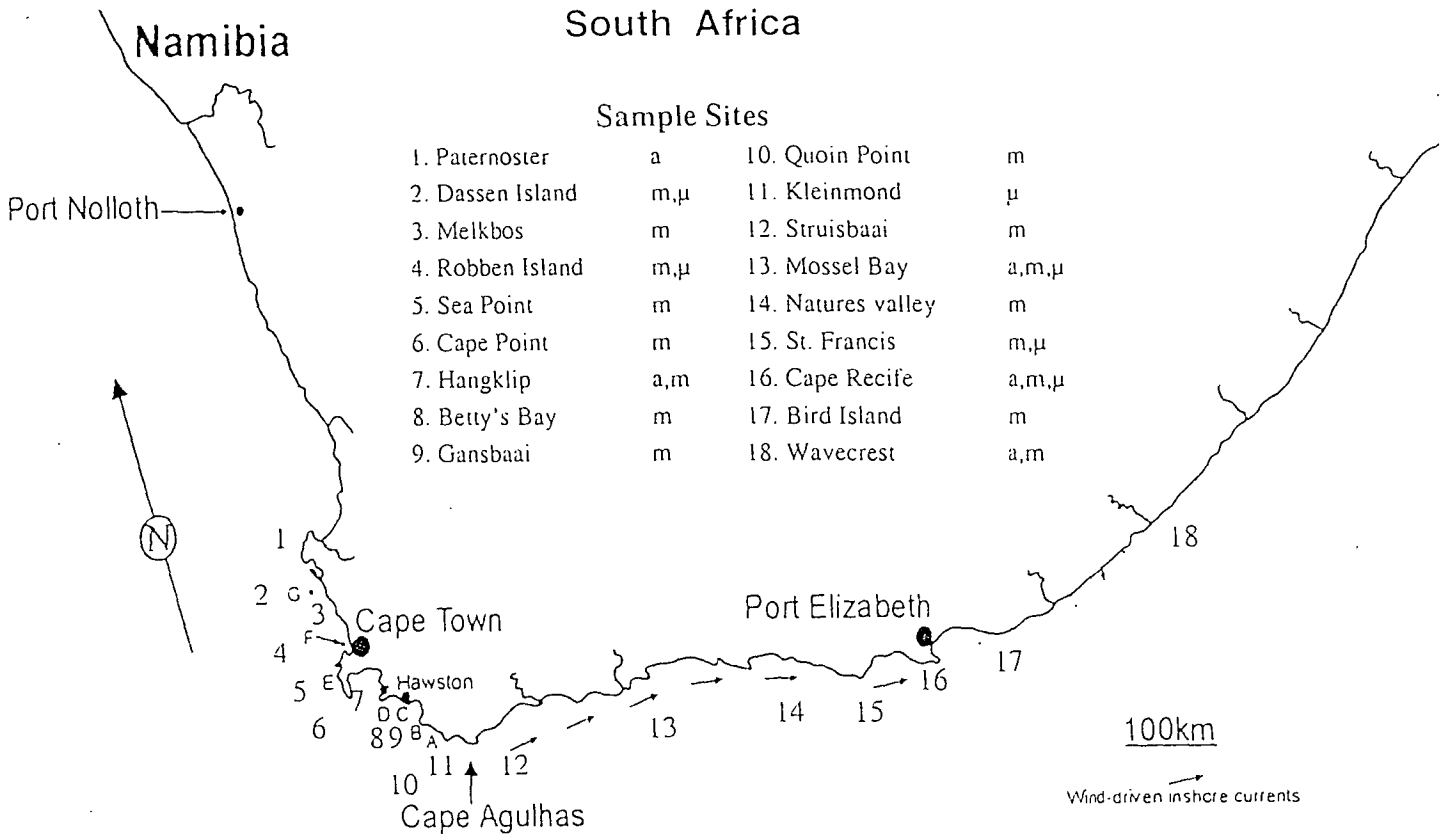

Figure 6-1 Collection sites for *H.midae* samples on the Cape Coast of South Africa. a and m indicate sites examined for allozyme and mitochondrial DNA variation (Sweijd 1999). μ indicates those sites included in the microsatellite analysis. Commercial fishing zones shown as A to G.

Island (DI), Robben Island (RI), Kleinmond (KL), Mossel Bay (MB), St Francis Bay (SF), Cape Recife (CR). Sample sizes for each locality are indicated in Table 6-1. Samples of gill tissue were obtained in good condition by separating all soft tissue from the shell and meats during processing, individual samples were sealed in bags and transferred to the University of Cape Town laboratory on ice. Gill tissue was isolated using sterile instruments to prevent cross-contamination of samples, and frozen at –20° C until required.

6.2.2 DNA Extraction

Total DNA was extracted from all samples using a CTAB extraction method (Grewe *et al.* 1993). Routinely, 50 mg of gill tissue was dissected and coarsely diced with a scalpel blade. The tissue was incubated at 65° C for 30 min in 2 mL of extraction buffer (100 mM Tris, 50 mM EDTA, 400 mM NaCl, 1% SDS pH 8) prior to the addition of proteinase K to a final concentration of 0.5 $mg.mL^{-1}$ and incubation at 55° C overnight. In order to differentially extract mucopolysaccharides (which otherwise co-precipitate with the DNA), 300 µL 0.75 M NaCl and 1% (v/v) CTAB solution (10% in 0.7 M NaCl) was added. Samples were then incubated at 65° C for 1 hr and then extracted with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated by addition of 2.5 volumes of ice-cold 100% ethanol. The suspension was centrifuged at 16 000 x g for 15 min to pellet the DNA, which was then washed in 70% ethanol. After air drying, the DNA was resuspended in 300 µL TE buffer and stored at 4° C until further analysis.

6.2.3 PCR amplification of microsatellite DNA

The development of the primer pairs (*CmrHr* 2.15, *CmrHr* 2.23 and *CmrHr* 2.29 GenBank accession numbers; AF195956, AF302832, AF302834), their optimization in other species and the characterization of their amplification products has been published (Evans *et al.* In Press; Chapter 3).

Three microsatellite markers (*CmrHr* 2.15, *CmrHr* 2.23 and *CmrHr* 2.29) were optimized for amplification in a Hybaid 96-well thermocycler. The amplification conditions began with an initial denaturation at 94° C for 3 min followed by 30 cycles of denaturation at 94° C (60 s), annealing at 51° (*CmrHr* 2.23) or 53° C (*CmrHr* 2.15 and *CmrHr* 2.29) (45 s), and extension at 72° C (60 s). Cycling was followed by a 6 min extension step at 72° C. Reactions were performed in a volume of 25 µL consisting of approximately 200 ng genomic DNA template, 200 µM of each dNTP: dTTP, dATP, dGTP, dCTP, 7 pM of each primer, either 1.0 mM (*CmrHr* 2.23) or 3.0 mM (*CmrHr* 2.15 and *CmrHr* 2.29) $MgCl_2$, 2 units of *Taq* DNA polymerase (Fisher Biotech) and ddH_20 to volume. A volume of 5 µL of each PCR product was electrophoresed on 1% (w/v in TAE buffer) agarose gels containing ethidium bromide

and visualised by UV transillumination to determine whether the reactions were successful. The intensity of these amplifications was used as a guide for dilutions prior to visualization on the ABI-373 fluorescence system.

Two microlitres from each amplified microsatellite locus was mixed and diluted to a final volume of 80 µL. Two microlitres of this dilution was then mixed with formamide, loading dye and Genescan Tamra-500 size standard (ABI), denatured at 95° C for 2 min, and loaded onto a 4% denaturing polyacrylamide gel. Samples were run on an ABI-373 DNA autosequencer and genotypes determined with Genotyper® software.

6.2.4 Statistical Analysis

Genetic diversity for each locus per sample site was estimated by the number of alleles per locus and by the observed (H_o) and Hardy-Weinberg expected (H_e) heterozygosity. H_o, H_e, and tests for deviations from Hardy-Weinberg Equilibrium (HWE) within samples were estimated using GENEPOP Vers. 3.2 (Raymond and Roussett 1995). An index of heterozygote deficiency or excess (D), where $D = [H_o-H_e] / H_e$ (Selander 1970) was also calculated from the heterozygosity estimates. Significance levels for deviations from HWE were based on 100 000 steps of a Markov chain procedure.

Linkage disequilibrium was assessed using exact tests in GENEPOP Vers. 3.2 (Raymond and Roussett 1995). Significance of departure from equilibrium levels was tested by a Markov chain procedure, with significance levels determined after 400 batches of 4000 iterations each.

The level of allele and genotypic frequency differences at each locus among abalone samples was assessed using exact tests in GENEPOP Vers. 3.2 (Raymond and Roussett 1995) with an unbiased estimate of *P* determined after 1000 batches of 10000 iterations each of a Markov chain. Significance was determined by a sequential Bonferroni procedure (Hochberg 1988) in order to correct for multiple tests.

Estimation of F-statistics (F_{IS}, F_{IT} and F_{ST}) was undertaken in GENEPOP Vers. 3.2 (Raymond and Roussett 1995). F_{ST} values for each locus were estimated by a weighted analysis of variance (Cockerham 1973; Weir and Cockerham 1984). Where overall estimates of F-statistics were significant, population pairwise estimates were obtained to identify the source of the differentiation.

ARLEQUIN Ver. 2.000 (Schneider *et al.* 2000) was used for an analysis of variance of allele frequencies within and among populations (AMOVA), a method based on Excoffier *et al.* (1993). ARLEQUIN also permitted multi-locus estimates of Φ_{ST}, an analogue of F_{ST}, the

proportion of the total genetic variation attributable to population differentiation. F-statistics were originally developed by Wright (1951) to assess population structure in terms of inbreeding coefficients. The fixation index F_{ST} is the same as the weighted average of F_{ST} over loci defined by Weir and Cockerham (1984).

Levels of genetic differentiation among populations were also calculated using R_{ST} (as Rho). R_{ST} is an analogue of F_{ST}, which has been specifically developed for the analysis of microsatellite data. It utilizes a stepwise mutation model (Slatkin 1995) and considers such parameters as variance in allele size and relatively high mutation rates. The RSTcalc package (Goodman 1997) was used to calculate Rho, an unbiased estimator of Slatkin's R_{ST} that corrects for potential biases that may result from unequal sample sizes and loci with unequal variances.

POPGENE Ver. 1.21. (Yeh *et al.* 1997) was used to produce an UPGMA dendogram, based on Nei's (1972) Genetic Distance and modified from the NEIGHBOUR procedure of PHYLIP version 3.5.

6.3 Results

The three microsatellite loci used were perfect dinucleotide (CA) repeat motifs. Allele frequencies are presented as Appendix B. Nineteen alleles were detected at locus *CmrHr* 2.15, two at *CmrHr* 2.23 and 17 at *CmrHr* 2.29.

Genetic diversity statistics were estimated by the number of alleles per locus and observed and Hardy-Weinberg expected heterozygosity per locus and per sample (Table 6-1). All loci were polymorphic in each of the populations examined. Mean numbers of alleles per locus per population ranged from two at locus *CmrHr* 2.23 to 9 at locus *CmrHr* 2.29, in sample sizes ranging from 51 (Cape Recife) to 21 (Dassen Is.) abalone per sample site. Mean observed heterozygosities per locus per population ranged from 0.188 (*CmrHr* 2.23) to 0.554 (*CmrHr* 2.29). (Table 6-1). Observed locus heterozygosities ranged from 0.167 (*CmrHr* 2.23 at DI) to 0.627 (*CmrHr* 2.29 at CR) (Table 6-1). A comparison of allele frequencies between pooled western and eastern samples revealed a significant decline in the number of alleles detected in the eastern samples. Mean number of alleles detected across all loci in the pooled western sample ($\Sigma n = 99$) was 12.3, whilst in the eastern group, with a slightly larger total sample size ($\Sigma n = 106$), an average of only 7.7 alleles were detected.

Linkage disequilibrium was assessed and no significant departure from equilibrium levels was detected in any sample.

Table 6-1 Genetic diversity estimates for South African *Haliotis midae* populations. [*N* sample size; N_{allele} number of alleles, *Mean* N_{allele} mean number of alleles per population; H_o observed heterozygosity; H_e expected heterozygosity; *D* Selander's index of heterozygote defficiency, negative values indicates an excess of homozygotes. *P* probability of deviation from Hardy-Weinberg equilibrium. **Significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction for multiple tests across loci. *All loci* provides mean values, with the exception of N_{allele} which is the sum of alleles across loci. *All loci P* value is calculated by combining probabilities across loci, and significance determined by comparison to critical values of chi-squared in Sokal and Rohlf (1981)].

Group	Population		*CmrHr* 2.15	*CmrHr* 2.23	*CmrHr* 2.29	*All loci*
All	Total	*N*	199	205	205	203
		N_{allele}	19	2	17	38
		Mean N_{allele}	9	2	7.8	12.7
		H_o	0.429	0.188	0.554	0.390
		H_e	0.669	0.243	0.674	0.529
		P	< 0.001**	0.993	0.006**	< 0.001**
	Dassen Is.	*N*	*21*	*24*	*24*	*23.0*
		N_{allele}	8	2	7	17
		H_o	0.333	0.167	0.500	0.333
		H_e	0.742	0.194	0.703	0.546
		P	0.003**	1.000	0.031	<0.010**
		D	-0.551	-0.139	-0.289	-0.326
West	*Robben Is*	*N*	*26*	*26*	*26*	*26.0*
		N_{allele}	11	2	4	17
		H_o	0.462	0.192	0.462	0.372
		H_e	0.776	0.212	0.596	0.528
		P	0.001**	1.000	0.026	<0.010**
		D	-0.405	-0.094	-0.225	-0.241
	Kleinmond	*N*	*48*	*49*	*49*	*48.7*
		N_{allele}	13	2	17	32
		H_o	0.396	0.245	0.612	0.418
		H_e	0.781	0.217	0.702	0.567
		P	< 0.001**	1.000	0.280	< 0.001**
		D	-0.493	0.129	-0.128	-0.164
	Mossel bay	*N*	*29*	*29*	*29*	*29.0*
		N_{allele}	7	2	7	16
		H_o	0.414	0.207	0.621	0.414
		H_e	0.512	0.220	0.766	0.499
		P	0.215	1.000	0.030	> 0.100
		D	-0.191	-0.059	-0.189	-0.147
East	*St. Francis*	*N*	*24*	*26*	*26*	*25.3*
		N_{allele}	5	2	4	11
		H_o	0.458	0.231	0.500	0.396
		H_e	0.560	0.348	0.563	0.490
		P	0.069	0.201	0.927	> 0.100
		D	-0.182	-0.336	-0.112	-0.210
	Cape Recife	*N*	*51*	*51*	*51*	*51.0*
		N_{allele}	10	2	8	20
		H_o	0.510	0.275	0.627	0.471
		H_e	0.641	0.267	0.712	0.540
		P	0.004	1.000	0.135	< 0.025**
		D	-0.204	0.030	-0.119	-0.098

Genotype proportions in each population for each locus were tested for goodness-of-fit to Hardy-Weinberg expectations (Table 6-1). Three of the 18 tests differed significantly from the Hardy-Weinberg expectations after sequential Bonferroni correction. Tests that showed significant deviations from Hardy-Weinberg expectations were for *CmrHr* 2.15 in the three western samples of; Dassen Is., Robben Is. and Kleinmond; all showed a significant excess of homozygotes ($P < 0.008$). Negative values of D (Selander 1970) were obtained for 16 of the 18 tests, indicating an overall deficit of heterozygotes (Table 6-1).

The significance of allele frequency differences, as calculated by exact tests in GENEPOP Vers. 3.2 (Raymond and Roussett 1995), are < 0.001 at locus *CmrHr* 2.15, 0.551 at locus *CmrHr* 2.23, and 0.001 at locus *CmrHr* 2.29, suggesting highly significant differentiation across the six sample sites. Population-pairwise exact tests across all loci show significant differences between five population pairs; Mossel Bay & the three western samples of Dassen Island ($P = 0.001$), Robben Island ($P < 0.001$), and Kleinmond ($P < 0.001$); St. Francis & Robben Island ($P = 0.001$); Kleinmond & Cape Recife ($P = 0.001$). Large shifts in the frequency of some alleles were detected at two loci. The *CmrHr* 2.15, 277 bp allele was present at a frequency of 0.374 in the western samples, and at the much higher frequency of 0.615 in the east. Likewise, the 426 bp allele at *CmrHr* 2.29 increased from a frequency of 0.248 in the west, to 0.373 in the east. These changes are not countered by opposing shifts in flanking allele frequencies and so are unlikely to be due to the incorrect scoring of alleles. No significant difference was detected within the eastern or western groups.

Estimates of overall F-statistics (F_{IS}, F_{IT} and F_{ST}), and R_{ST} are presented in Table 6-2 and range from the negative F_{ST} value of - 0.002 at *CmrHr* 2.23 indicating completely mixed stocks, to the relatively high level of 0.034 at *CmrHr* 2.15. An overall estimate of F_{ST} across the three loci and six populations is 0.024, whilst R_{ST} is 0.039. These values correlate closely to that obtained by the AMOVA method of Excoffier *et al.* (1993) which provides a multi-locus estimate of Φ_{ST}, an analogue of F_{ST}, at 0.027 ($P < 0.001$).

Sample pairwise F_{ST} values are presented in Table 6-3 and range from a minimum of – 0.002 for the comparison of Robben Island and Kleinmond samples, to a maximum of 0.067 for the Robben Island and St Francis samples. Five population pairs are significantly different after sequential Bonferroni correction, they are: Kleinmond and the three east coast samples of Mossel Bay ($P < 0.001$), St. Francis ($P < 0.001$), and Cape Recife ($P = 0.002$); Robben Island and both Mossel Bay ($P < 0.001$) and St. Francis ($P < 0.001$).

Table 6-2 Overall estimates of F-statistics. F_{IS}, F_{ST} , F_{IT} estimated using GENEPOP vers.3.2. R_{ST} estimated using RSTcalc. and Φ_{ST} estimated using ARLEQUIN version 2.000

Locus	N	$N_{alleles}$	F_{IS}	F_{ST}	F_{IT}	R_{ST}	Φ_{ST}
*CmrHr*2.15	199	19	0.343	0.034	0.365	0.089	0.039
*CmrHr*2.23	205	2	-0.014	-0.002	-0.016	-0.003	-0.002
*CmrHr*2.29	205	17	0.152	0.023	0.172	0.032	0.025
All	203	12.7	0.209	0.024	0.228	0.039	0.027

Table 6-3 Sample-pairwise R_{ST} values, presented as Rho below diagonal, population-pairwise F_{ST} values above diagonal **Significant pairwise differentiation after sequential Bonferroni correction.

	Dassen Is.	Robben Is.	Kleinmond	Mossel Bay	St. Francis	Cape Recife
Dassen Island	----------	0.001	0.002	0.030	0.024	0.001
Robben Island	0.019	----------	-0.002	0.065**	0.067**	0.022
Kleinmond	0.013	-0.011	----------	0.067**	0.059**	0.028**
Mossel Bay	0.019	0.059**	0.055**	----------	0.026	0.004
St. Francis	0.058	0.133**	0.111**	0.027	----------	0.012
Cape Recife	0.000	0.035**	0.024	0.000	0.028	----------

Population-pairwise R_{ST} values are presented in Table 6-3 as Rho. These values range from –0.011 for the comparison of Robben Island and Kleinmond samples, to a maximum of 0.133 for the Robben Island and St Francis samples. Five population pairs are significantly different after sequential Bonferroni correction; Robben Island and the three East coast samples of Mossel Bay ($P < 0.001$), St. Francis ($P < 0.001$) and Cape Recife ($P < 0.001$);Kleinmond and both Mossel Bay ($P < 0.001$) and St. Francis ($P < 0.001$).

No significant F_{ST} or R_{ST} differences were detected within the three east-coast populations, or within the three west-coast samples (Table 6-3). These samples were therefore pooled into the two groups divided geographically by Cape Agulhas and a second AMOVA performed (Table 6-4). This produced a highly significant ($P < 0.001$) Φ_{CT} value of 0.033 across all loci.

Nei's (1972) genetic distance measurements are represented in an UPGMA dendogram (Figure 6-2). The pairwise values range from a minimum of 0.013 between Kleinmond and Robben Island to a maximum of 0.096 between Robben Island and St. Francis. The dendogram divides the populations into the west and east groups as suggested by the population-pairwise comparisons.

Table 6-4 Analysis of Molecular Variance comparing a group of 3 samples to the west of Cape Agulhas to a group of 3 samples east of that point. Estimates of Φ_{CT} obtained using Arlequin version 2.000 **Significant differentiation after sequential Bonferroni correction for multiple tests.

Locus	N	$N_{alleles}$	Φ_{CT}
CmrHr 2.15	199	19	0.059**
CmrHr 2.23	205	2	0.006
CmrHr 2.29	205	17	0.013**
All	203	12.7	0.033**

6.4 Discussion

Previous research into the genetic structure of abalone populations has yielded variable results. Strong genetic structuring has been reported at three allozyme loci for seven *H. cracherodii* sample sites (N_{mean} = 57) along the central Californian coast (Hamm and Burton 2000), by mitochondrial DNA data in *H. diversicolor* from five sites (N_{mean} = 12) around Taiwan (Jiang *et al.* 1995), and at six RAPD and three microsatellite loci for 10 *H. rubra* sample sites (N_{mean} = 10) in southern Australia (Huang *et al.* 2000). Weaker structure was detected at multiple allozyme loci in *H. roei* (Hancock 2000 – 10 sites, N_{mean} = 48) and *H. rubra* (Brown 1991 – 23 sites, N_{mean} = 62) populations in Australia. Studies of *H. fulgens* in Baja California revealed no signs of genetic structuring at seven allozyme loci (Zúñiga *et al.* 2000 – 5 sites, N_{mean} = 20), as was the case at four allozyme loci for *H. rufescens* populations (3 sites, N_{mean} = 154) along the Californian coast (Gaffney *et al.* 1996; Burton and Tegner 2000). Chapter 4 of this thesis presents evidence of restricted gene flow between *H. rubra* samples around Tasmania, and those around mainland Australia (10 sites, N_{mean} = 82)

Sweijd (1999) found measures of average heterozygosity at seven allozyme loci in *H. midae* to be high ($H_{e\ mean}$ = 0.311), but within the range of published data for abalone species. In this study I report an average heterozygosity (H_e) across three microsatellite loci in six samples (*N* = ~33) of *H. midae* of 0.529. This value is low compared to values of average H_e reported at microsatellite loci in other abalone species: H_e = 0.908 (Huang *et al.* 2000; 3 loci, 10 samples, *N* = 10) and 0.756 (Conod *et al.* In Press; 5 loci, 5 samples, *N* = 100) in *H. rubra*; H_e = 0.757 in *H. rufescens* (Kirby *et al.* 1998; 1 locus, 2 samples, *N* = 35); H_e = 0.626 in *H. laevigata* (Personal Communication: Natalie Conod, CSIRO Marine research, Hobart; 3 loci, 6 samples, *N* = 30). One possible explanation for the low levels of heterozygosity seen in this study is the use of microsatellite markers that were designed in the related species, *Haliotis rubra*. The fact that these markers have been conserved in abalone species other than the one they were designed for may suggest that they are located in more conserved regions of the genome. This theory is supported by the lower heterozygosity detected in *H. laevigata* samples, again examined using markers designed for *H. rubra*.

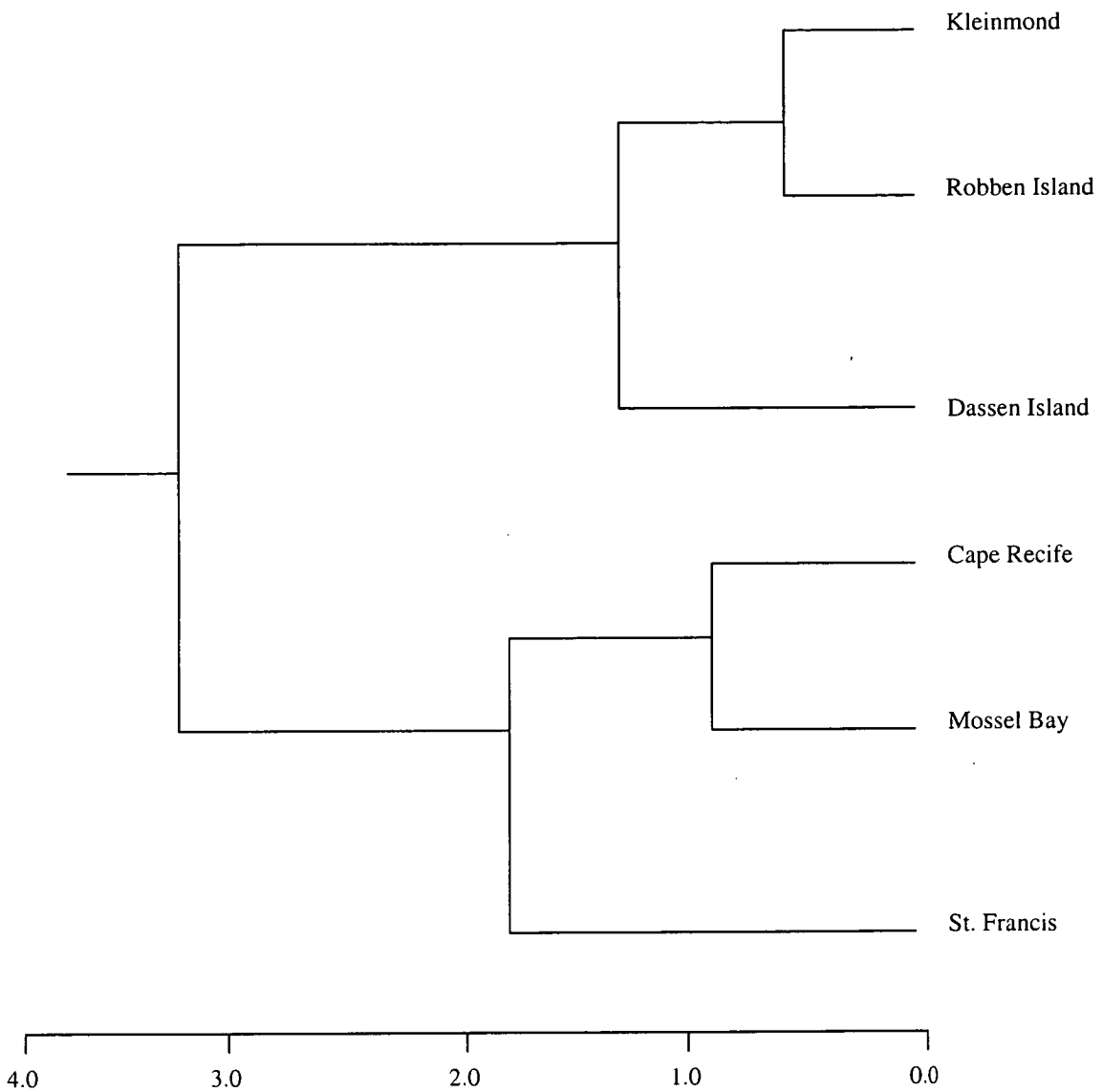

Figure 6-2 UPGMA dendogram of Nei's (1972) genetic distances

All significant departures from Hardy-Weinberg equilibrium were the result of heterozygote deficiencies, which are not uncommon in marine molluscs (Zouros and Pogson 1994; Chapter 4). In this case, significant deviations were seen only at the *CmrHr* 2.15 locus and were not consistent across samples, they are therefore unlikely to be explained by inbreeding or by the Wahlund effect (Richardson 1982). No significant deviation from HW proportions was found in the *H.midae* populations studied by Sweijd (1999) using allozyme markers. Numerous reasons have been cited for the common appearance of heterozygote excess in microsatellite studies; a common suggestion is the presence of null or non-amplifying alleles (Chapter 4 and 5). These are difficult to detect in wild populations, without the aid of parentage studies, but will lead to an apparent excess of homozygosity. Null alleles can be caused by mutations within the primer sites of microsatellite loci. Primer site mutations have been identified by some researchers (Ede and Crawford 1995; Eggleston-Stott *et al.* 1997; Chapter 5), but are often just assumed to be the cause of amplification failure (for example: McGoldrick *et al.* 2000). The confirmation of null allele presence at microsatellite loci in blacklip abalone populations around Tasmania (Chapter 5) suggests that the homozygote excess detected in four *H. midae* samples at locus *CmrHr* 2.15 could be due to null alleles.

The microsatellite data presented here shows no significant difference in heterozygosity across samples or between east and west-coast samples. A reduction in the number of alleles was detected in the pooled eastern sample as compared to the western sample. This is consistent with previous mtDNA findings of a reduction in the genetic diversity of *H. midae* populations to the east of Cape Agulhas (Sweijd 1999).

The mtDNA data reported by Sweijd (1999), and the microsatellite data presented here show significant genetic structure within populations of *H. midae* along the coast of South Africa. The probability of differentiation as calculated by exact tests (GENEPOP Ver. 3.2) was highly significant ($P < 0.005$) at two of the three loci examined. The overall F_{ST} value produced by GENEPOP was 0.024 ($P < 0.001$), and Φ_{ST} as calculated by the AMOVA method of ARLEQUIN (ver. 2.000) was 0.028 ($P < 0.001$). All these tests assume the infinite-allele model of microsatellite mutation, where alleles identical in state are identical by descent. This approach has been criticized as being less representative of microsatellite mutation than the stepwise mutation model (SMM) proposed by Slatkin (1995). Debate continues as to the best model for use in microsatellite based population analyses, and so measures based on each model are presented for comparison here.

Rho is a measure of R_{ST} that accounts for differences in sample size and allelic variance, and is based on the assumption of the SMM. The SMM states that mutations derived by these models will cause allelic states to switch backwards and forwards by small increments (Di Renzo *et al.* 1994), and most new alleles will be disguised as pre-existing allelic states. As a result, F_{ST}

values derived from microsatellite data may be underestimating true levels of genetic differentiation (Goldstein *et al.* 1995). Population-pairwise values for Rho estimated here suggest significant differentiation between five east and west-coast samples. Whilst not all east-coast samples were significantly differentiated from all west-coast samples, no differentiation was detected within the eastern or the western sample groups (Table 6-4).

In sample-pairwise F_{ST} and exact test comparisons in both ARLEQUIN (Ver 2.000) and GENEPOP (ver. 3.2) respectively, significant differences were consistently found between east and west-coast samples, but none within either group (Table 6-3). This homogeneity of east and west sites allowed the division of samples into these two groups for AMOVA analysis which revealed a highly significant Φ_{CT} value of 0.040 ($P < 0.001$). Similar values of Φ_{ST} have been described at allozyme loci in *H. cracherodii* samples along the central Californian coast ($F_{ST} = 0.039$) (Hamm and Burton 2000), however, this differentiation was not supported by mtDNA data collected from the same samples. In my study the microsatellite data do lend strong support to the dramatic discontinuity described by mtDNA analysis by Sweijd (1999), who found a Φ_{CT} value of 0.387 for a comparison of eastern and western samples.

Estimates of genetic distance are represented in an UPGMA dendogram, which clearly divides the six samples into two groups, one from either side of Cape Agulhas, further supporting the east-west division. Within the groups however there appears to be no relationship between genetic distance and geographic distance.

Sweijd (1999) proposed that the eastern populations of *H. midae* are the result of an isolated introduction from established populations in the west, and a subsequent easterly range expansion. This hypothesis is strongly supported by his mtDNA data and by the microsatellite data presented here, but some explanation is required to ensure the allozyme data is interpreted correctly.

In a single introduction event, with all markers being selectively neutral, we would expect mitochondrial DNA analysis to show the strongest signs of a founder effect due to maternal inheritance and haploid nature combining to reduce its effective population size (Avise 1994). Both nuclear markers (allozymes and microsatellites) are expected to have an effective population size four times greater than mtDNA (Avise 1994). If the easterly populations were then reproductively isolated from those to the west of Cape Agulhas for a sufficient time period, the two groups would diverge through processes of drift and mutation. The mutation rate of each of the markers is such that we could expect microsatellite markers, which are presumed to have the highest mutation rate (Freeland *et al.* 2000), to quickly begin to show signs of differentiation between the two groups. This rapid mutation rate could also be

expected to ensure that any loss of heterozygosity in populations to the east would be quickly hidden by the relatively fast allele frequency changes that result. Because microsatellite mutation may simply produce an existing allelic state, rather than a new allele each time, a founder population may take longer to recover from a loss of alleles, than the associated loss of heterozygosity. On average, mitochondrial DNA markers evolve at a slower rate than microsatellites, but faster than allozymes (Bowen *et al.* 1993; Avise 1994; Schlötterer 2000). Mitochondrial DNA markers evolve quite rapidly, and would be expected to show divergence between the two groups in the form of haplotype frequency changes, increases in frequency of some haplotypes (Grewe *et al.* 1993), the creation of new haplotypes, and the loss of some haplotypes during the introduction (Ovenden and White 1990). The loss of diversity at the initial introduction due to the low effective population size of mtDNA would be expected to be very high. Due to the high mutation rate of the mtDNA molecule though, diversity levels could be expected to recover over a time frame somewhat longer than that seen in the microsatellite data, but shorter than at allozyme loci. Allozymes have the lowest mutation rate of any of these three marker classes, and would be expected to be the least likely to show any genetic differentiation over a short time frame. They would be expected to undergo some loss of genetic diversity during an isolated introduction, although their higher initial effective population size would ensure that the loss was not to the same extent seen in mtDNA data. Therefore, we could expect to see this type of depressed genetic diversity maintained at allozyme loci over short to medium time frames such as the one hypothesised here.

In the case of *Haliotis midae* presented here, the above description of a hypothetical founder event and subsequent range expansion is supported by all the available data collected from three forms of genetic marker. Only the consistently high levels of heterozygosity at allozyme loci across the range of *H. midae* is inconsistent with the hypothesis, although no significant differentiation was detected at allozyme loci.

Other studies have produced conflicting patterns of genetic differentiation with different genetic markers. Karl and Avise (1992) cite numerous examples where data from allozyme loci indicate little or no population subdivision, only for mtDNA studies to later reveal discontinuities (see Ovenden and White 1990 for a dramatic example). Karl and Avise (1992) suggest that the lack of differentiation at allozyme loci can be attributed to balancing selection on protein coding loci. They reach this conclusion by revealing discontinuities at nuclear genes similar to those described by mtDNA data in oysters, despite a complete lack of population subdivision detected at allozyme loci in the same samples. Their conclusion emphasizes the need for caution when inferring population genetic structure and gene flow from any single class of genetic marker.

Burton (1996) concludes that the choice of a molecular technique to a given problem should be based on its efficiency. Sweijd (1999) has previously shown the efficiency of the PCR-RFLP approach of mtDNA data to infer population structure in *H. midae*. He recommended further research using alternative markers to examine fine scale population structure, and as a way to test the disparity between diversity levels (in the east and west) revealed by allozymes and mtDNA. The microsatellite markers used here have supported the differentiation of the east and west coast stocks of *H. midae*, but have not revealed any fine scale population subdivision within those two zones, suggesting that populations on either side of Cape Agulhas represent two independent reproductive stocks. A significant reduction in the number of alleles was detected at microsatellite loci in the combined eastern samples, and large allele frequency changes, often associated with founder events (Allendorf and Ryman 1988), were also detected between the western and eastern groups. Evidence from allozyme, mitochondrial DNA and microsatellite data supports an isolated introduction event to the east of Cape Agulhas, and subsequent range expansion in an easterly direction as originally proposed by Sweijd (1999). The disparity between allozyme data and the other two forms is seen as further evidence for the presence of balancing selection (as described by Karl and Avise 1992) at allozyme loci.

Chapter 7 Abalone culture- application of genetic markers

7.1 Introduction

Microsatellite DNA markers have a variety of uses within aquaculture research. Microsatellites are non-coding, highly polymorphic, co-dominant DNA markers. These qualities make them a powerful tool to measure the response of hatchery populations to different breeding strategies in terms of changes in genetic variation. Microsatellites are also useful for the identification of closely related individuals and for parentage analysis. In more established industries such as livestock production, microsatellites are used in conjunction with other molecular markers to develop genetic maps. These maps can then be used to facilitate the identification of quantitative trait loci (QTLs), which are central to the development of marker assisted selection (MAS) programs.

Abalone production in hatcheries and farms is increasing around the world, for both direct commercial gain, and for re-seeding of native stocks (Hahn 1989). The high fecundity (> 1 million eggs per female) of mature abalone, and artificially high survival rate of juvenile abalone in a culture environment ensures that sufficient seed for each year's production may result from only a small number of parents. When small numbers of broodstock are used, or related individuals are mated, there may be a subsequent decrease in genetic variability of farmed stocks (Norris *et al.* 1999). Such problems are amplified when hatchery reared broodstock are used outside of a genetically controlled breeding program (Hahn 1989). Loss of genetic variation at allozyme loci has been reported in hatchery populations of salmonids subject to multiple generations of culture (Ryman and Stahl 1980, Vuorinen 1984), and also in first generation hatchery populations (Verspoor 1988). More recent microsatellite analysis of wild and farmed Atlantic salmon populations revealed a decrease in genetic variability of farmed stocks in terms of allelic diversity but not in overall heterozygosity (Norris *et al.* 1999).

Previous studies of diversity in first generation hatchery stocks of abalone have utilised allozyme electrophoresis (Smith and Conroy 1992; Mgaya *et al.* 1995; Gaffney *et al.* 1996). These studies have all reported the absence of alleles that are rare in the wild population from the farmed samples, although the findings of Gaffney *et al.* (1996) have since been disputed by Burton and Tegner (2000). Smith and Conroy (1992) report a significant reduction in heterozygosity, a loss of rare alleles and significant shifts in allele frequency at two polymorphic allozyme loci in hatchery reared *Haliotis iris*. An examination of genetic variation at three polymorphic allozyme loci in first and third generation hatchery reared *Haliotis tuberculata* revealed a loss of rare alleles when the third generation stock was

compared to the first generation and the wild samples (Mgaya *et al.* 1995). No evidence of inbreeding or reduction of heterozygosity was reported.

Such reductions in genetic variation have been shown to be detrimental to commercially important traits such as growth rate (Koehn *et al.* 1988) and fitness (Danzmann *et al.* 1989) in other marine organisms. Reductions in the genetic variation of abalone cultured for re-seeding operations could have a negative impact on native stocks in the area of re-seeding by reducing genetic variation or swamping locally adapted genotypes (Allendorf and Ryman 1988).

Chapters 4 and 6 of this thesis outline the genetic variation present at microsatellite loci within wild samples of the Australian blacklip abalone, *Haliotis rubra*, and the South African Perlemoen, *Haliotis midae*. Whilst these are not the first studies to investigate genetic variation in wild samples of abalone as revealed by microsatellite markers (see Kirby *et al.* 1998; Huang *et al.* 2000), they are the most comprehensive in terms of sample size and number of loci examined. This chapter is the first study to employ microsatellite markers to compare levels of genetic variation in farmed and wild samples of abalone. Such changes in genetic variation may be measured as a shift in the frequency of common alleles, as a loss of rare alleles, and as a reduction in mean heterozygosity through loss of common alleles (Smith and Conroy 1992).

Here I present a comparison of variability at three microsatellite loci within and between two samples of farmed South African abalone and six wild samples from the extent of the species range, the two farmed samples are from either side of the Cape Agulhas. Levels of genetic variation in the six wild samples of *H. midae* were reported in Chapter 6, with significant differentiation observed between samples from either side of Cape Agulhas. I also investigate variation at five microsatellite loci within and between four cohorts of farmed Tasmanian abalone and compare these with that observed in seven wild samples from around Tasmania. Levels of genetic variation within natural stocks of Australian blacklip abalone were investigated at eight microsatellite loci in Chapter 4. In addition, the inheritance of two microsatellite markers is analysed in three *H. rubra*, *H. laevigata* hybrid abalone family lines to evaluate the use of microsatellite markers for pedigree analysis in hybrid abalone. The inheritance of two microsatellite loci was also assessed.

7.2 Methods

Intellectual property issues associated with the respective abalone farms restrict the information pertaining to the spawning and husbandry conditions of both sets of hatchery abalone. No information was available as to the number of broodstock used, the number of

isolated spawning events or the occurrence of grading or mixing of stocks. The general geographic area of broodstock collection was however made available by each farm.

7.2.1 Samples

7.2.1.1 South African hatchery abalone

Fifty *Haliotis midae* individuals from a single year class were obtained from a commercial abalone farm on the south-west coast of South Africa (western farm). The parent stock were originally obtained from a mixture of Western Cape wild populations. The sample consisted of a randomly selected group of 6-month old juveniles that were obtained from a single growout tank, and represent progeny from multiple broodstock.

A further 50 *H. midae* individuals were collected from a commercial abalone farm on the east-coast of South Africa, near Port Elizabeth (eastern farm). This sample comprised 6-month old juveniles from multiple broodstock obtained from Cape Recife on the east coast.

All cultured abalone collected in South Africa were transported live from the farm to the University of Cape Town. They were then individually dissected using sterile instruments to isolate gill tissue, which was stored at -20° C until DNA extraction.

Details of the six wild samples of *H. midae* examined are presented in Chapter 6.

7.2.1.2 Tasmanian hatchery abalone

Sixty-four *Haliotis rubra* juveniles were collected from each of four commercial raceways at a Tasmanian abalone farm. The individuals were all removed from a single randomly selected artificial substrate in each raceway. Each raceway is representative of an isolated cohort (separate multiple broodstock spawning) of individuals settled between five and 8 months earlier.

Cultured *H. rubra* individuals were transported on ice to CSIRO Marine Research, Hobart, where they were individually dissected using sterile instruments to isolate gill tissue, which was then stored at -20° C until DNA extraction.

Details of the seven Tasmanian wild samples of *H. rubra* examined are presented in Chapter 4.

7.2.1.3 Tasmanian hybrid cohorts

Between October and December 1998 spawning attempts were made on 12 nights to produce single parent crosses, or family lines of *H. rubra* (female), *H. laevigata* (male) F1 hybrid abalone from wild caught broodstock. Spawning and nursery facilities were established in a small scale research facility on the premises of a commercial abalone culture operation in Tasmania.

Haliotis laevigata broodstock were collected by commercial divers from sites along the North-East coast of Tasmania. *Haliotis rubra* broodstock were collected from sites on the Tasman Peninsula, South-Eastern Tasmania. Animals selected as broodstock were in healthy condition and had very ripe gonads. Gonad ripeness was gauged by the raised posture of the abalone on the substrate, and an obvious swelling of the gonads, particularly at the distal end which appeared rounded in ripe animals, rather than its normal pointed appearance. All animals were transported to the commercial facility in mesh bags suspended within a 300 L container of seawater. Aeration was supplied by the direct addition of oxygen to the water from an oxygen cylinder.

Standard hatchery procedures were observed (Hone *et al.* 1997), with fertilisation, hatching and larval growth undertaken on a commercial scale. The broodstock were held in tanks for between one and three days before spawning was attempted. On each of 12 nights, 8 to 15 greenlip male broodstock and a similar number of blacklip female broodstock were arranged in two tiers with females isolated in individual containers along the top row, and males in individual containers beneath them. To stimulate spawning, a constant flow of filtered, UV treated sea-water was supplied to the top tier of animals and the overflow was directed into the containers below. The UV breaks down the oxygen in the seawater to ozone which is thought to trigger spawning by stimulating the production of PG-endoperoxide in the reproductive system, which in turn, boosts the secretion of the hormone prostaglandin (Uki 1989).

Immediately after any spawning activity was detected the UV source was switched off, and flow-through ceased. Released ova and sperm were collected by siphoning and mixed in a jug at a ratio of between five and ten sperm per egg. After ten minutes the eggs were rinsed through a wet sieve to remove excess sperm before being distributed evenly within the commercial hatching tank. After approximately 24 hours the majority of viable eggs had hatched and larvae had moved towards the surface of the tank. These were siphoned off into large upwelling tanks where the larvae developed over six to ten days. After this time they started to display a foot testing response prior to settlement.

The larvae from six successful spawnings were settled onto pre-conditioned plastic settlement plates. A random sample of approximately 10 000 larvae from each family were settled onto 8

plates within individual 120 L tanks. After an initial settlement time of two days these tanks were supplied with aeration and a constant flow-through of filtered seawater. Poor settlement success and very high mortality in three tanks reduced the numbers of progeny to below 10 individuals in each cross. Some mortality was associated with water filtration problems at the facility, leading to a decline in water quality in all tanks. The differential survival in family lines may be due to variable tolerances to environmental conditions, and this in turn could be genetically controlled. Unfortunately, no DNA could be obtained from the dead juveniles for comparison. Each of the three remaining family lines were maintained in these tanks until sampled at age 18 months. Sampled abalone had been off the plates and fed on artificial diets for at least six months before sampling.

Simultaneous spawning of both male greenlip and female blacklip abalone was achieved on seven occasions. In one case the fertilization rate was effectively zero and all eggs were discarded. Reasons for such an event are unclear, although sperm from the same male was used on the same night for the successful production of a hybrid family with another female. Both family 4 and 5 were thought to have been produced as single pair crosses, although records show that it was unclear which broodstock had been involved in the spawning.

The production of hybrid individuals for this research led to difficulties that would not have been expected in single species production. Difficulties began with an inability to make the spawning times of greenlip males and blacklip females coincide. Large numbers of eggs were produced on two instances when no sperm could be induced from males. On two more occasions, quantities of sperm were produced when no female could be induced to spawn. The environmental factors associated with the induction of abalone spawning are little understood. It is likely that differences in these conditions will favour the spawning of one species in preference to the other, and research into these factors is currently underway in the blacklip abalone (Personal Communication: Mark Grubert, PhD. candidate, abalone broodstock conditioning, Tasmanian Aquaculture and Fisheries Institute).

In order to test the pedigree of the family lines produced, a non-lethal tissue sample was taken from the spawned broodstock for DNA extraction and typing. This was achieved by the removal of one or both cephalic tentacles from each broodstock individual that spawned. Tentacles were transported to the laboratory in 1.5 mL microfuge tubes on ice, and stored at -20 °C prior to DNA extraction.

The sampled juvenile *H. rubra / H. laevigata* hybrid individuals were transported on ice to CSIRO Marine Research, Hobart, where they were individually dissected using sterile instruments to isolate muscle tissue, which was then stored at -20° C until DNA extraction.

7.2.2 DNA Extraction

DNA was extracted from a single cephalic tentacle from each broodstock individual by a modified CTAB procedure (Grewe *et al.* 1993). Commercial DNA extraction kits (QIAGEN) were used to extract DNA from muscle tissue from the smaller cultured individuals, as these samples produced low quality DNA from the CTAB procedure.

7.2.3 PCR amplification of microsatellite loci

7.2.3.1 South African hatchery abalone

Three microsatellite markers (*CmrHr* 2.15, *CmrHr* 2.23 and *CmrHr* 2.29) were optimized for amplification in a Hybaid 96-well thermocycler. The amplification conditions, data collection and analysis of microsatellite variation in the South African samples is outlined in Chapter 6. The development of the primer pairs (*CmrHr* 2.15, *CmrHr* 2.23 and *CmrHr* 2.29: GenBank accession numbers; AF195956, AF302832, AF302834), their optimization in other species and the characterization of their amplification products has been published (Evans *et al.* In Press; Chapter 3).

7.2.3.2 Tasmanian hatchery abalone

Five of the eight microsatellite markers used to investigate *Haliotis rubra* population differentiation in Chapter 4 were used to investigate diversity in the four hatchery produced cohorts. The markers used were: *CmrHr* 1.14, *CmrHr* 2.14, *CmrHr* 2.30, *CmrHr* 1.24 and *RUBCA1* (GenBank accession numbers: AF195952, AF195957, AF195959, AF195953, AF027573). These five loci were amplified together in a single multiplex PCR reaction and genotypes were determined on an ABI 377 as described in Chapter 4.

7.2.3.3 Tasmanian hybrid cohorts

Only two *Haliotis rubra* microsatellite loci, *CmrHr* 2.14 and *CmrHr* 2.30 (GenBank accession numbers: AF195957, AF195959) produced reliable amplification products in both parent species and the hybrid progeny. All broodstock that spawned and 24 randomly selected progeny from each of the three family lines were PCR amplified and genotyped for the two loci. In the case of Family 2, a further 48 progeny were included in the analysis at a later time.

PCR reactions were performed in a volume of 25 µl consisting of 67 mM TrisHCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg.mL^{-1} gelatin; 2.5 mM $MgCl_2$; 10 pmoles of each primer; 200 µM dNTPs; 0.5 U *Taq* F1 polymerase (Fisher Biotech); and ~ 20 ng genomic DNA template. Amplification was in a Perkin Elmer 9600 thermocycler with an intial denaturation at 94° C for 3 min. Then 10 cycles of denaturation at 94° C for 15 s, annealing at

60° to 55° C for 15 s (- 0.5° C cycle^{-1}) and extension at 72° C for 1 min., followed by a further 25 cycles of 55° C annealing. These cycles were followed by a final extension step of 72° C for 10 min. Genotypes were determined by electrophoresis of amplification products on 4.8% polyacrylamide gels on the ABI 377 DNA autosequencer, and analysis using (Genotyper Ver 1.1) software.

7.2.4 Statistical analysis

Genetic diversity for each locus per farm cohort was estimated, and compared to the diversity in wild samples of the same species. Diversity was measured by the number of alleles per locus and by the observed (H_o) and Hardy-Weinberg expected (H_e) heterozygosity. H_o, H_e, and tests for deviations from Hardy-Weinberg Equilibrium (HWE) within samples were estimated using GENEPOP Vers. 3.2 (Raymond and Roussett 1995). An index of heterozygote deficiency or excess (D), where $D = [H_o-H_e] / H_e$ (Selander 1970) was also calculated from the heterozygosity estimates. Significance levels for deviations from HWE were based on 100 000 steps of a Markov chain procedure.

Linkage disequilibrium was assessed in each of the three sample groups using exact tests in GENEPOP Vers. 3.2 (Raymond and Roussett 1995). Significance of departure from equilibrium levels was tested by a Markov chain procedure, with significance levels determined after 400 batches of 4000 iterations each.

ARLEQUIN Vers. 2.000 (Schneider *et al.* 2000) was used for an analysis of variance of allele frequencies within and among samples, as well as between all sample pairs (AMOVA) in *H. rubra* and *H. midae* investigations. The method is based on Excoffier *et al.* (1993). ARLEQUIN also permitted multi-locus estimates of Φ_{ST}, an analogue of F_{ST}, the proportion of the total genetic variation attributable to population differentiation.

POPGENE Ver. 1.21. (Yeh *et al.* 1997) was used to produce an UPGMA dendogram for *Haliotis midae* samples. These dendograms are based on Nei's (1972) Genetic Distance and modified from the NEIGHBOUR procedure of PHYLIP version 3.5.

7.3 Results

7.3.1 South African hatchery abalone

Allele frequencies of farmed and wild caught *H. midae* are presented as Appendix B. Within the two farmed samples thirteen alleles were detected at locus *CmrHr* 2.15 (11 in western farm, seven in eastern farm), two at *CmrHr* 2.23 and 11 at *CmrHr* 2.29 (ten in western farm, five in eastern farm). Two previously unrecorded alleles were identified at locus *CmrHr* 2.15,

one in each of the farmed samples, both were high repeat alleles at frequencies of 0.051 and 0.010. A single allele at *CmrHr* 2.29 was recorded in the east farm sample (frequency of 0.010) that was not observed in the parental sample site at Cape Recife. Common alleles in the wild remained common in the farm samples.

All loci were polymorphic in each of the farmed samples examined. Numbers of alleles per locus can provide an accurate measure of genetic variation when the samples being compared are similar in sample size. To that end, each of the farmed samples was compared to a single wild sample from the area of broodstock collection, containing similar sample sizes (Table 7-1). A loss of eight of twenty alleles (40%) was observed in the eastern farm sample from that in the wild sample of Cape Recife. All lost alleles were at a frequency of less than 0.07 in the wild sample. A loss of 12 of 32 alleles (37.5%) was observed in the western farm sample when compared to the Kleinmond sample. All lost alleles were present at a frequency of less than 0.06 in the wild sample. Observed heterozygosities per locus were similar in all samples with the exception of locus *CmrHr* 2.29 in the western farm sample which was higher than all wild samples at $H_o = 0.800$. This compares to values of 0.612 and 0.627 at this locus in the Kleinmond and Cape Recife samples (Table 7-1).

Linkage disequilibrium was assessed and no significant departure from equilibrium levels was detected in any sample.

Genotype proportions in the farmed samples were tested for goodness-of-fit to Hardy-Weinberg expectations at each locus (Table 7-1). Three of the six tests differed significantly from the Hardy-Weinberg expectations after sequential Bonferroni correction. Those tests that showed significant deviation from Hardy-Weinberg expectations were for *CmrHr* 2.15 in both farmed samples and *CmrHr* 2.29 in the eastern farm sample. All significant deviations from Hardy-Weinberg expected heterozygosities were due to an excess of homozygotes. Negative values of *D* (Selander 1970) were obtained for 5 of the 6 tests, indicating an overall deficit of heterozygotes (Table 7-1).

Population pairwise F_{ST} values were calculated between the east and west farm samples and pooled wild samples from either side of Cape Agulhas. Significant differentiation was observed between all pairs ($P < 0.001$) with the exception of the pooled western Cape sample and the western farm sample. The western samples were not differentiated as demonstrated by the F_{ST} value of 0.000 for that comparison. The eastern farm sample was significantly differentiated from the pooled eastern wild sample (Table 7-2).

Table 7-1 Genetic diversity estimates for South African hatchery *Haliotis midae* samples. [*N* sample size; N_{allele} number of alleles, H_o observed heterozygosity; H_e expected heterozygosity; *D* Selander's index of heterozygote defficiency, negative values indicates an excess of homozygotes. *P* probability of deviation from Hardy-Weinberg equilibrium. **Significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction for multiple tests across loci. *All loci* provides mean values, with the exception of N_{allele} which is the sum of alleles across loci. *All loci P* value is calculated by combining probabilities across loci, and significance determined by comparison to critical values of chi-squared in Sokal and Rohlf (1981)].

Sample		*CmrHr* 2.15	*CmrHr* 2.23	*CmrHr* 2.29	*All loci*
Kleinmond.	*N*	*48*	*49*	*49*	*48.7*
	N_{allele}	13	2	17	32
	H_o	0.396	0.245	0.612	0.418
	H_e	0.781	0.217	0.702	0.567
	P	< 0.001**	1.000	0.280	< 0.001**
	D	-0.493	0.129	-0.128	-0.164
West Farm	*N*	*49*	*49*	*50*	*49.3*
	N_{allele}	11	2	10	23
	H_o	0.408	0.204	0.800	0.471
	H_e	0.767	0.239	0.712	0.573
	P	< 0.001**	1.000	0.703	< 0.001**
	D	-0.468	-0.146	0.124	-0.164
Cape Recife	*N*	*51*	*51*	*51*	*51*
	N_{allele}	10	2	8	20
	H_o	0.510	0.275	0.627	0.396
	H_e	0.641	0.267	0.712	0.490
	P	0.004**	1.000	0.135	< 0.025**
	D	-0.204	0.030	-0.119	-0.210
East Farm	*N*	*48*	*50*	*50*	*49.3*
	N_{allele}	7	2	5	14
	H_o	0.417	0.340	0.520	0.426
	H_e	0.596	0.393	0.535	0.508
	P	< 0.001**	0.323	0.012**	< 0.001**
	D	-0.300	-0.135	-0.028	-0.154

Nei's (1972) genetic distance measurements are represented in an UPGMA dendogram (Figure 7-1). The pairwise values range from a minimum of 0.011 between eastern farm and St Francis to a maximum of 0.102 between the two farmed samples. The pairwise distance measurements between eastern farm and St. Francis (0.011) and between western farm and Dassen Island (0.011) are the two lowest measured across all samples examined. The dendogram places the farmed populations into their respective group representing the broodstock from either side of Cape Agulhas.

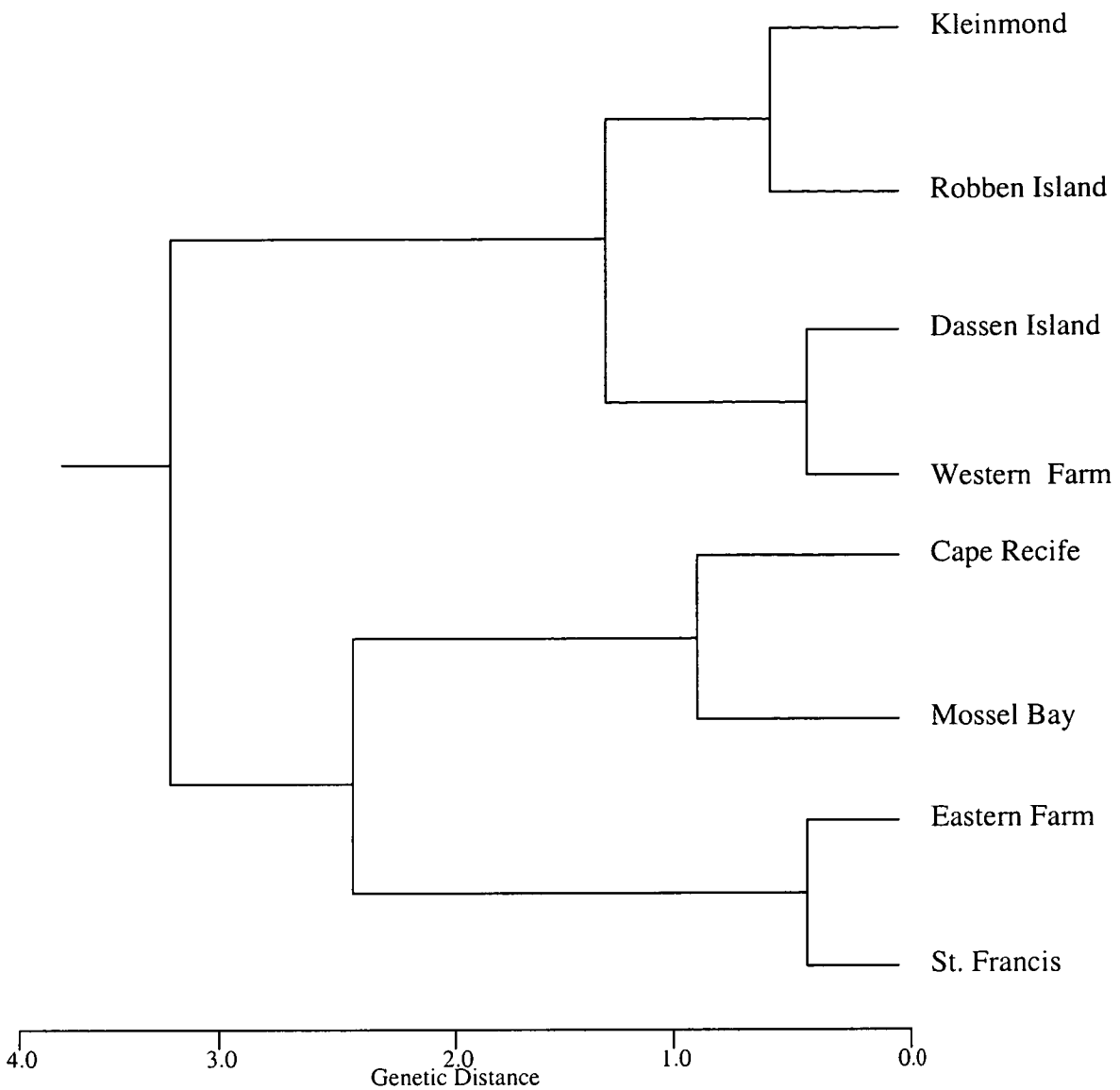

Figure 7-1 UPGMA dendogram of Nei's (1972) genetic distances between samples of wild and farmed *H. midae*.

Table 7-2 Pairwise F_{ST} values for comparison of farmed samples of *Haliotis midae* to pooled wild samples. ** reflects significant differentiation after sequential Bonferroni correction. A complete list of pairwise differences within the wild samples is provided in Chapter 5.

	West Farm		East Farm	
	F_{ST}	*P*	F_{ST}	*P*
Wild west pooled	0.000	0.541	0.060**	< 0.001
West Farm	----------	----------	0.041**	< 0.001
Wild east pooled	0.022**	0.002	0.031**	< 0.001
East farm	0.041**	< 0.001	----------	----------

7.3.2 Tasmanian hatchery abalone

A complete list of allele frequencies are presented as Appendix C. To ensure that sample sizes were similar for allele number comparisons, each farm raceway (N = 64) was compared to the wild samples of George Third Reef (N = 68) and Trumpeter Corner (N = 61). No significant differentiation had been found between samples from around Tasmania (Table 7-4 and Chapter 4), therefore these two samples were representative of the natural population, from which the broodstock had been collected. The total number of alleles observed across five loci in the two wild samples was 83 in the Trumpeter Corner sample and 84 in the George Third Reef sample. Total number of alleles declined in each of the four raceway samples. The largest loss of alleles (62%) occurred from the George Third Reef sample (Nallele = 84) to the Raceway 2 sample (Nallele = 32), with the smallest loss of alleles (35%) being from the Trumpeter Corner sample (Nallele = 83) to the Raceway 4 sample (Nallele = 54) (Table 7-3). Because all raceways were representative samples of a single year class in one hatchery, they were pooled to create a single farm sample (N = 256) which showed a 35% reduction in alleles from the similar sized grouping of wild samples from Georges Rock, Curio Bay and George Third Reef (N = 260). All alleles lost to the farm samples were present at frequencies of less than 0.09 in all wild samples.

At the highly polymorphic locus *CmrHr* 2.30 (60 alleles in wild samples) no new alleles were observed in the farm samples. Three previously unrecorded alleles were observed at *rubCA1* (all in Raceway 2), and one at each of the other three loci. In Raceway 2, the new 124 bp allele at *rubCA1* was observed at a frequency of 0.349, making it the most common allele at this locus. In three of the four Raceways, the most common allele observed at *rubCA1* was at a higher frequency than it occured in wild samples. More common alleles were similar in farmed and wild samples for *CmrHr* 1.14, *CmrHr* 1.24 and *CmrHr* 2.14. At locus *CmrHr* 2.30 each of the four Raceways had a different more common allele (frequency of > 0.170), which in each case were at low frequencies in the wild samples.

All loci with the exception of *CmrHr* 1.24 were polymorphic in all samples. Only the 222 bp allele was present at this locus in the Raceway 2 sample, this allele is at a frequency of greater than 0.70 in all other samples. Observed heterozygosity per locus ranged from 0.000 at that monomorphic locus, to 0.984 at *CmrHr* 2.30 in Raceway 1 (Table 7-3). When the raceway samples were pooled to provide a single farm sample for comparison, the average observed heterozygosity was 0.574, compared to a value of 0.578 in the pooled wild sample.

Linkage disequilibrium was assessed and no significant departure from equilibrium levels was detected in any sample.

Genotype proportions in all farmed samples were tested for goodness-of-fit to Hardy-Weinberg expectations at each locus (Table 7-3). Ten of the 20 tests differed significantly from the Hardy-Weinberg expectations after sequential Bonferroni correction. All samples showed significant departures from the expected at the *rubCA*1 and *CmrHr* 2.30 loci, while all samples were in Hardy-Weinberg equilibrium at the *CmrHr* 1.14 and *CmrHr* 1.24 loci. The Raceway 2 and Raceway 4 samples both differed significantly from Hardy-Weinberg expected values at *CmrHr* 2.14. All significant deviations from Hardy-Weinberg expected heterozygosities in the wild samples were due to an excess of homozygotes (Table 7-3). In the farmed samples however, five of the ten significant values represent an excess of heterozygotes. Negative values of *D* (Selander 1970) were obtained for all wild samples, indicating an overall deficit of heterozygotes (D_{wild} = - 0.128) in natural populations. Three farmed samples produced positive *D* values, indicating an excess of heterozygotes. The *D* value for the pooled farmed samples was - 0.049 suggesting a slight excess of homozygotes across all loci in the farm stocks (Table 7-3).

The AMOVA method of Excoffier *et al.* (1993) was used to provide a multi-locus estimate of Φ_{ST}, an analogue of F_{ST}, of 0.044 ($P < 0.0001$) across all samples. Population pairwise F_{ST} values indicate that the differentiation is derived from within the Raceway samples, with all Raceway samples significantly different to all wild samples and all other Raceway samples (data not shown). No significant differentiation between wild samples was detected after sequential Bonferroni correction (Table 7-4). Raceway and wild samples were then grouped separately and compared in a second AMOVA. The F_{CT} value, a measure of differentiation attributable to differences between the two groups was 0.013 ($P < 0.001$), and the F_{SC} value, a measure of the differentiation attributable to differences between the samples within those groups was 0.038 ($P < 0.001$). Because the Raceway samples were all representative of a single year class within the farm, they were pooled as a single sample and the pairwise F_{ST} test was run again. Pooling of samples that are genetically differentiated is not statistically acceptable in wild samples, but is valid in this case as all animals are essentially from a single farm stock. The combined farm sample was significantly different to each wild sample ($P < 0.001$; Table 7-4).

7.3.3 Tasmanian hybrid cohorts

The genotypes of eight *Haliotis laevigata* males, and 12 *H. rubra* females that spawned at one of seven successful spawning events are provided in Table 7-5. The genotypes of 24 individuals from each of the three successful family lines were determined to confirm parentage (Tables 7-6a, 7-7, 7-8). Only Family 2 (Table 7-6a, b) was shown to be a true family line upon genotypic analysis, with the purported parents, blacklip female 4, and greenlip male 1 being confirmed as the correct parents, as only those alleles expected were observed.

Table 7-3 Genetic diversity estimates for Tasmanian *Haliotis rubra* samples. [*N* sample size; N_{allele} number of alleles, H_o observed heterozygosity; H_e expected heterozygosity; *P* probability of deviation from Hardy-Weinberg equilibrium.**Significant departure from Hardy-Weinberg expected equilibrium after sequential Bonferroni correction for multiple tests across loci. *All loci* provides mean values, with the exception of N_{allele} which is the sum of alleles across loci. *All loci P* value is calculated by combining probabilities across loci, and significance determined by comparison to critical values of chi-squared in Sokal and Rohlf (1981)]

Population		*rubCA*1	*CmrHr* 1.14	*CmrHr* 1.24	*CmrHr* 2.14	*CmrHr* 2.30	All
Georges Rocks	*N*	*100*	*100*	*100*	*94*	*100*	*98.8*
	N_{allele}	34	9	6	13	49	105
	H_o	0.790	0.360	0.350	0.585	0.860	0.589
	H_e	0.887	0.410	0.333	0.658	0.970	0.652
	P	0.686	0.012	0.944	0.091	0.110	< 0.050**
	D	-0.109	-0.122	0.051	-0.111	-0.113	-0.096
Trump. Corner	*N*	*61*	*61*	*61*	*61*	*60*	*60.8*
	N_{allele}	32	5	5	7	34	83
	H_o	0.918	0.328	0.377	0.623	0.850	0.619
	H_e	0.915	0.386	0.411	0.708	0.955	0.675
	P	0.241	0.145	0.729	0.548	0.077	> 0.100
	D	0.003	-0.150	-0.083	-0.120	-0.110	-0.083
One tree point	*N*	*86*	*91*	*90*	*72*	*89*	*85.6*
	N_{allele}	33	7	7	7	42	96
	H_o	0.86	0.253	0.378	0.569	0.674	0.547
	H_e	0.894	0.231	0.357	0.671	0.958	0.622
	P	0.166	1.000	0.711	0.377	0.003**	> 0.050
	D	-0.038	0.095	0.0589	-0.152	-0.296	-0.121
Curio Bay	*N*	*92*	*92*	*91*	*81*	*87*	*88.6*
	N_{allele}	31	7	5	7	41	91
	H_o	0.848	0.272	0.275	0.827	0.690	0.582
	H_e	0.905	0.310	0.317	0.738	0.954	0.645
	P	0.176	0.005**	0.298	0.025	< 0.001**	< 0.001**
	D	-0.063	-0.123	-0.132	0.121	-0.277	-0.097
George III Reef	*N*	*61*	*62*	*66*	*62*	*61*	*62.4*
	N_{allele}	28	6	5	9	36	84
	H_o	0.787	0.323	0.197	0.597	0.672	0.515
	H_e	0.942	0.410	0.211	0.739	0.959	0.652
	P	< 0.001**	0.082	0.549	0.009**	< 0.001**	< 0.001**
	D	-0.165	-0.212	-0.066	-0.192	-0.299	-0.210
Sterile Island	*N*	*87*	*90*	*92*	*80*	*79*	*85.6*
	N_{allele}	31	7	6	9	42	95
	H_o	0.828	0.300	0.293	0.663	0.722	0.561
	H_e	0.893	0.356	0.306	0.740	0.962	0.651
	P	0.002**	0.137	0.770	0.159	0.031	< 0.010**
	D	-0.073	-0.157	-0.042	-0.104	-0.249	-0.138
Church Rocks	*N*	*96*	*95*	*96*	*92*	*96*	*95.0*
	N_{allele}	30	7	5	8	47	97
	H_o	0.875	0.400	0.323	0.685	0.833	0.560
	H_e	0.897	0.392	0.352	0.695	0.964	0.660
	P	0.647	0.431	0.276	0.266	< 0.001**	< 0.001**
	D	-0.025	0.020	-0.082	-0.014	-0.136	-0.151

Table 7-3 Continued..

Population		*rubCA* 1	*CmrHr* 1.14	*CmrHr* 1.24	*CmrHr* 2.14	*CmrHr* 2.30	All
Raceway 1	*N*	*64*	*64*	*64*	*64*	*64*	*64.0*
	N_{allele}	11	2	5	5	14	37
	H_o	0.891	0.047	0.328	0.688	0.984	0.588
	H_e	0.832	0.091	0.289	0.551	0.857	0.524
	P	< 0.001**	0.077	0.838	0.204	< 0.001**	< 0.001**
	D	0.071	-0.487	0.135	0.249	0.149	0.121
Raceway 2	*N*	*63*	*64*	*64*	*64*	*64*	*63.8*
	N_{allele}	14	4	1	6	7	32
	H_o	0.714	0.500	-	0.922	0.969	0.776
	H_e	0.820	0.551	-	0.644	0.733	0.687
	P	< 0.001**	1.000	-	< 0.001**	< 0.001**	< 0.001**
	D	-0.129	-0.093	-	0.432	0.322	0.130
Raceway 3	*N*	*63*	*64*	*64*	*63*	*63*	*63.4*
	N_{allele}	12	3	3	6	16	40
	H_o	0.698	0.453	0.031	0.587	0.968	0.547
	H_e	0.763	0.389	0.062	0.571	0.851	0.527
	P	< 0.001**	0.132	1.000	0.267	< 0.001**	< 0.001**
	D	-0.085	0.165	-0.500	0.028	0.137	0.038
Raceway 4	*N*	*64*	*64*	*64*	*63*	*64*	*63.8*
	N_{allele}	18	5	4	6	21	54
	H_o	0.906	0.141	0.219	0.619	0.797	0.536
	H_e	0.930	0.189	0.217	0.744	0.919	0.600
	P	< 0.001**	0.090	0.164	0.003**	< 0.001**	< 0.001**
	D	-0.026	-0.254	0.009	-0.168	-0.133	-0.106
Farm sample	*N*	*254*	*256*	*256*	*254*	*255*	*255.0*
	N_{allele}	30	6	6	8	30	80
	H_o	0.803	0.285	0.145	0.705	0.929	0.574
	H_e	0.935	0.347	0.141	0.653	0.940	0.603
	P	< 0.001**	< 0.001**	0.802	0.010**	< 0.001**	< 0.001**
	D	-0.141	-0.179	0.028	0.080	-0.011	-0.049
Wild sample	*N*	*583*	*591*	*596*	*542*	*572*	*576.8*
	N_{allele}	43	13	8	10	60	134
	H_o	0.842	0.320	0.315	0.653	0.760	0.578
	H_e	0.902	0.349	0.321	0.703	0.967	0.648
	P	0.146	< 0.001**	0.868	< 0.001**	< 0.001**	< 0.001**
	D	-0.067	-0.083	-0.019	-0.071	-0.214	-0.109

Table 7-4 Population-pairwise F_{ST} values for seven wild Tasmanian samples and one farm sample (4 raceway samples combined) of *Haliotis rubra* at 5 microsatellite loci (below diagonal). *P* values are given above the diagonal for a null hypothesis of no differentiation between samples. Values in bold are significant at $P < 0.05$ ** those tests still significant after sequential Bonferroni correction for multiple tests.

	Georges Rocks	*Trump. Corner*	*Curio Bay*	*One Tree Point*	*George III Reef*	*Sterile Island*	*Church Rocks*	*Tas Ab Farms*
Georges Rocks	----------	0.321	0.485	**0.010**	**0.006**	0.845	0.519	**0.000**
Trump. Corner	0.001	----------	0.949	0.403	**0.038**	0.570	0.939	**0.000**
Curio Bay	0.000	-0.003	----------	0.213	**0.012**	0.746	0.607	**0.000**
One Tree Point	0.005	0.001	0.001	----------	**0.002**	0.088	0.273	**0.000**
George III Reef	0.007	0.006	0.007	0.008	----------	0.165	**0.003**	**0.000**
Sterile Island	-0.001	0.000	-0.001	0.003	0.003	----------	0.370	**0.000**
Church Rocks	0.000	-0.002	0.000	0.001	0.007	0.001	----------	**0.000**
Tas Ab farms	0.021**	0.023**	0.020**	0.020**	0.019**	0.020**	0.027**	----------

Both Family 4 and 5 contain only 2 individuals each in the 24 examined that could be the progeny of the two putative parents (Tables 7-7 and 7-8). Twenty different genotypes were recorded in the progeny of each family.

In Family 4 there are three greenlip (male parental) and three blacklip (female parental) alleles at *CmrHr* 2.14 and four greenlip (male parental) and six blacklip (female parental) alleles at *CmrHr* 2.30. Two alleles are present in the progeny of this family that were not seen in any of the genotyped broodstock spawned at the same time as these family lines, they are the 291 bp allele at *CmrHr* 2.14 and a 377 bp allele at *CmrHr* 2.30.

Family 5 contains three greenlip (male parental) and four blacklip (female parental) alleles at *CmrHr* 2.14, and four greenlip (male parental) and ten blacklip (female parental) alleles at *CmrHr* 2.30 (Table 6-8). Three alleles recorded at *CmrHr* 2.30 in the progeny of this family were not present in the broodstock surveyed, they are the 339, 357 and 377 bp alleles, all of which are larger than the largest allele seen in the sampled broodstock (333 bp), but within the range of blacklip alleles reported for the population structure in Chapter 4. No null homozygotes were observed at either locus. The presence of null heterozygotes in broodstock is unlikely given that none of the progeny in any family appeared to be homozygous for blacklip or greenlip alleles.

As Family 2 was confirmed as a true family line, a further 48 progeny were genotyped. Of the 72 individuals typed, only 5 contained the *CmrHr* 2.30, 236 bp allele of the greenlip male parent. All other alleles were present in proportions commensurate with Mendelian inheritance (Table 7-6b).

Table 7-5 Genotypes of wild caught broodstock spawned during endeavors to produce *H. rubra*, *H. laevigata* hybrid family lines. Alleles sizes are in base pairs. Presumed parents of a) Family 2, b) Family 4, c) Family 5.

	Broodstock	*CmrHr* 2.14		*CmrHr* 2.30	
a	greenlip male 1	279	283	236	242
	greenlip male 2	279	283	242	242
	greenlip male 3	279	279	242	247
c	greenlip male 4	275	275	236	242
b	greenlip male 5	283	287	242	242
	greenlip male 6	279	279	236	236
	greenlip female 7	279	279	242	244
	greenlip male 8	283	283	240	247
	blacklip female 1	200	224	299	309
	blacklip female 2	224	236	299	323
	blacklip female 3	224	236	299	329
a	blacklip female 4	224	236	290	311
c	blacklip female 5	216	224	292	305
b	blacklip female 6	216	224	330	333
	blacklip female 7	224	224	304	315
	blacklip female 8	212	236	324	329
	blacklip female 9	224	224	310	329
	blacklip female 10	228	236	309	309
	blacklip female 11	224	224	306	311
	blacklip female 12	236	236	299	299

Table 7-6a Family 2. Parental genotypes and frequency of occurrence of each of the possible genotypes in 72 hybrid progeny.

	CmrHr 2.14		*CmrHr* 2.30		
blacklip female 4	224	236	291	311	number of times
greenlip male 1	279	283	236	242	seen in progeny
	224 279		291 242		8
	224 279		311 242		9
	224 283		291 242		11
	224 283		311 242		11
	236 279		291 242		6
	236 279		311 242		6
	236 283		291 242		9
possible genotypes	236 283		311 242		7
	224 279		291 236		0
	224 279		311 236		1
	224 283.		291 236		0
	224 283		311 236		0
	236 279		291 236		2
	236 279		311 236		0
	236 283		291 236		2
	236 283		311 236		0
					72

Table 7-6b Family 2. Parental allele sizes and observed and expected (by Mendelian inheritance) frequency of occurrence in 72 hybrid progeny.

	CmrHr 2.14		*CmrHr* 2.30	
blacklip female allele (bp)	*224*	*236*	*291*	*311*
expected occurrence	36	36	36	36
observed occurrence	40	32	38	34
greenlip male allele (bp)	*279*	*283*	*236*	*242*
expected occurrence	36	36	36	36
observed occurrence	32	40	5	67

Table 7-7 Family 4. Putative parental genotypes and frequency of occurrence of each observed genotype in 24 hybrid progeny. * Genotypes matching expected parental alleles

Supposed Parents	*CmrHr* 2.14		*CmrHr* 2.30			
blacklip female 6	224	236	291	311	number of times	
greenlip male 5	279	279	242	244	seen in progeny	
	224 279		311 242		1	*
	224 279		299 242		1	
	224 283		291 242		1	
	228 279		309 240		2	
	228 279		377 240		1	
	228 279		377 240		1	
	228 279		309 244		1	
	228 291		309 244		3	
Observed Genotypes	236 279		309 240		1	
	236 279		299 242		1	
	236 279		299 242		1	
	236 279		329 242		1	
	236 279		291 242		1	*
	236 283		291 242		1	
	236 283		329 242		2	
	236 291		291 236		1	
	236 291		311 236		1	
	236 291		291 242		1	
	236 291		309 244		1	
	236 291		377 244		1	
					24	

Table 7-8 Family 5. Putative parental genotypes and frequency of occurrence of each observed genotype in 24 hybrid progeny. * Genotypes matching expected parental alleles.

	CmrHr 2.14		*CmrHr* 2.30			
blacklip female 5	216	224	293	305	number of times	
greenlip male 4	275	275	236	242	seen in progeny	
	216 275		293 236		1	*
	216 283		309 240		1	
	216 283		305 242		5	
	216 283		291 242		1	
	216 283		377 244		1	
	224 275		305 236		1	*
	224 275		377 240		1	
	224 275		329 242		1	
	224 283		309 240		1	
Observed Genotypes	224 283		339 242		1	
	224 283		357 242		1	
	224 283		291 242		1	
	224 287		293 242		1	
	224 287		309 244		1	
	228 283		305 242		1	
	228 283		299 242		1	
	228 283		311 242		1	
	236 283		311 236		1	
	236 283		357 242		1	
	236 283		291 242		1	
					24	

7.4 Discussion

7.4.1 Hatchery abalone

The five and three microsatellite loci used to investigate genetic variation in *Haliotis rubra* and *H. midae* respectively reveal genetic differences between wild and hatchery stocks in both species. This is evident as a loss of rare alleles across most loci in all hatchery samples when compared to wild stocks (35 - 62%), but not as a decline in overall heterozygosity. Tasmanian hatchery samples were genetically differentiated from all Tasmanian wild samples, whilst the structure of South African farm samples resembled wild samples from locations within the two areas of broodstock collection.

The loss of rare alleles from hatchery stocks has been reported as a more meaningful measure of genetic variation. This is because heterozygosity is insensitive to the substantial genetic changes that may occur in cultivated aquaculture stocks within the first generations of culture (Hedgecock and Sly 1990). In fact, Vuorinen (1984) states that the total extinction of any allele can be considered more harmful than a reduction in overall heterozygosity.

The loss of alleles from all of the first generation hatchery stocks examined in this study must therefore be considered as a significant genetic alteration of cultured stocks. The loss of these alleles from the first generation stocks reduces the variation available for future breeding programs using hatchery reared abalone as broodstock. Whilst there is a minimum of 35% reduction in allele numbers in all farm samples measured when compared to wild samples, this apparent decline in diversity of farm stocks is most likely exaggerated from real levels by the sampling methods employed. Each farm sample was collected from a single raceway, or grow-out tank, representing only a fraction of the total number of tanks at each farm. In the study of Tasmanian hatchery samples, four raceways were sampled, of more than 40 on site, with each individual raceway sample being produced in a different spawning event. By pooling allele frequencies from each of the four raceways sampled (Appendix C), I would expect to see a more realistic estimation of genetic variation within the total farm stock. When this is compared to a wild sample containing a similar number of individuals however, the reduction in rare alleles observed is approximately 35%. The number of grow-out tanks on each South African farm is not known, and therefore the proportion of the total farm stock sampled is also unclear.

Observed heterozygosity values of farmed and wild samples were similar over all loci (Table 7-1 and 7-3) in both species, with no apparent loss of heterozygosity in any of the farm samples measured. The loss of rare alleles without any noticeable reduction in heterozygosity can be an indication of a short term population bottleneck (Nei *et al.* 1975; Allendorf 1986) as would be the case during the foundation of farmed strains. Such findings have been reported at allozyme loci in the Pacific oyster, *Carassostrea gigas* (Hedgecock and Sly 1990) and brown trout, *Salmo trutta* (Vuorinen 1984) and at microsatellite loci in Atlantic salmon, *Salmo salar* (Norris *et al.* 1999) that have been maintained in culture through multiple generations. Mgaya *et al.* (1995) revealed no loss in heterozygosity at three allozyme loci in either first or third generation cultured European abalone, *Haliotis tuberculata*, when compared to wild samples. They did however reveal the loss of one allele in the first generation hatchery sample, and two alleles in the third generation sample when each was compared to the wild sample (9 alleles). Smith and Conroy (1992) also noted a loss of rare allozyme alleles at allozyme loci in a first generation hatchery sample of *H. iris*, which was associated with a reduction in heterozygosity at both allozyme loci examined. The highly polymorphic nature of microsatellites in abalone (N_{allele} locus^{-1} in *H. rubra* = 26.8) compared with allozymes (N_{allele} locus^{-1} in *H. rubra* = 6.1, Brown 1991) make them highly sensitive for measuring change in genetic variation between hatchery and wild samples.

Significant genetic differentiation was evident between wild samples of *H. midae* on either side of Cape Agulhas at three microsatellite loci as detailed in Chapter 6 of this thesis. Pairwise F_{ST} values and an UPGMA tree based on Nei's (1972) genetic distance measure

support the association of farmed samples with the wild samples in the areas from which broodstock were collected. The western farm sample was shown to be most similar to the wild sample at Dassen Island, a common region for broodstock collection in the western Cape, whilst the eastern farm sample was significantly differentiated ($P < 0.001$) from the wild sample at Cape Recife, the stated source of broodstock collection, but very similar to the St Francis sample ($P = 0.541$). No significant genetic differentiation was observed between wild samples of *H. rubra* from around Tasmania, whilst all Raceway samples, and a combined Tasmanian farm sample were genetically differentiated from all wild samples. Similar differentiation at allozyme loci has been reported between first generation hatchery and wild stocks of *H. iris* (Smith and Conroy 1992), and multiple generation hatchery and wild stocks of brown trout, *Salmo trutta* (Ryman and Stahl 1980).

This study demonstrates that there is a reduction in genetic variation in the first generation Tasmanian and South African abalone hatchery stocks examined when compared to appropriate wild samples. Minimizing this reduction is important to aquaculture, in that genetic diversity is the source of variation in important commercial traits such as growth rate and disease resistance (Vuorinen 1983). When that variation is lost in first generation hatchery stocks, it is lost to all subsequent generations within a closed breeding program, and may therefore limit the genetic improvement available within that stock.

Of perhaps more concern to industry is the change in frequency of some alleles, leading to change in the most common alleles observed. In Raceway 2 of the Tasmanian farm samples the *rubCA1* 124 bp allele is recorded at a frequency of 0.349, despite not being recorded in the wild samples (603 individuals). The extent of local adaptation in abalone is not well understood, and therefore any major change in allele frequencies could impact on the fitness of the species (Conover 1998) in future breeding programs, or in an area of restocking.

Current abalone farming practices in Tasmania and South Africa primarily utilise broodstock collected from the wild, and as such, the level of genetic variation in each successive year of production is independent of the last. This situation ensures that although a loss of variation may affect the survival and performance of one year class, it will not influence subsequent year classes. Theory suggests that the number of effective broodstock required to ensure that at least 99% of genetic variation is preserved is at least 50 individuals (Allendorf and Ryman 1988), although this could translate to more than double that number of broodstock used each year (Mgaya *et al.* 1995). The maintenance of genetic diversity becomes even more important when F1 animals are to be used as broodstock for subsequent generations. This is because any loss of diversity caused by poor farm practice, will be amplified in subsequent generations.

Smith and Conroy (1992) recommend that hatchery reared abalone should not be ongrown for use as adult broodstock, as this would compound any loss of genetic variation in the second generation. The reduction of variation in successive generations would be a serious problem for abalone culture, but should not prevent the closing of the breeding cycle within the hatchery. The gains in production that can be made through genetic breeding programs are up to 21% per generation (oysters; Bondari 1983), and the development of highly polymorphic DNA markers such as microsatellites enables the monitoring of genetic variation in each generation. Microsatellites can also be used to determine relatedness between potential broodstock and to identify the parentage of well performed progeny.

Rather than avoiding the use of hatchery reared broodstock, I would suggest that their use is a necessary step forwards for the industry. If hatchery reared broodstock are used as part of a well designed genetic breeding program such as those employed by the more established Atlantic salmon and cattle production industries (Gjerde 1986; Schrooten *et al.* 2000), then abalone culture production could be expected to increase rapidly in subsequent generations. A controlled genetic breeding program would facilitate these gains, whilst minimizing the loss of genetic variation attributed to selection for desirable traits.

Abalone re-seeding has been investigated as a means to increase numbers of abalone in South Africa (Sweijd 1999), and this has been trialled in an area that is now devoid of natural stocks, but which historically maintained healthy natural populations. Sweijd (1999) suggested that genetic variation at microsatellite loci be investigated as a tool for the identification of re-seeded stocks in areas already containing abalone. In my South African study it appears that the three markers available would be of limited use, given that the farmed samples were genetically similar to natural populations in their region, thus making any distinction between wild and re-seeded stocks unlikely. Re-seeding success could however be monitored effectively through the genotyping of broodstock and subsequent parentage analysis of re-seeded populations. Additional loci to the three used here would be needed to provide sufficient polymorphism for the unequivocal identification of juveniles. For this approach to be successful it would be suggested that further, highly polymorphic markers be developed. A suite of five or more markers with ~25 alleles each should provide sufficient power (probability of identity is 5.76×10^{-9} for 123 alleles; Perez-Enriquez and Taniguchi 1999). The suite of microsatellite markers used to investigate the structure of *Haliotis rubra* populations in Chapter 4 could most likely be used to individually identify each broodstock abalone used in a mass spawning. However, due to the presence of null alleles at some of these loci (eg. *CmrHr* 1.25 and *CmrHr* 2.30, see Chapter 5) it is recommended that other loci be investigated in order to avoid this technical problem. Areas that had been subject to re-seeding efforts could then be sub-sampled at intervals after settlement in order to determine the proportion of

individuals in that area that are the result of the re-seeding efforts. Such an approach would be similar in cost, but much more powerful than an analysis of genetic structure.

7.4.2 Tasmanian hybrid cohorts

The culture of abalone in Tasmania is a relatively new enterprise, with the first commercial venture only established approximately 15 years ago. One of the primary limiting factors of culture production in that time has been the ability to reliably induce wild caught broodstock to spawn on cue. Both ozone and hydrogen peroxide have been used as triggers for spawning, with varying success, and these factors in concert with controlled temperature shocks have been adopted by industry. Increased levels of ozone as a product of water treatment with ultraviolet light was the method used to induce spawning in this study. Simultaneous spawning was more difficult to achieve because I was attempting to spawn both *H. rubra* and *H. laevigata* and induction cues may vary between the two species.

The decision to produce hybrid family lines for this research was made in order to examine the amplification of microsatellite loci designed for use in one of the parental species, *H. rubra*, in the *H. rubra*, *H. laevigata* hybrid progeny. Hybrid abalone are produced commercially at one farm in Tasmania, and so I was able to draw on their experience for the production of my own family lines. Unfortunately, as stated earlier, both the production of hybrid families and the amplification of *H. rubra* microsatellite loci in *H. laevigata* parents and hybrid progeny proved problematic.

7.4.2.1 Family 2

As the only true family line produced, this family was examined for the presence of non-related progeny and spurious alleles, as observed in the other family tanks. Such presence could be a sign of sperm contamination at fertilisation, inadvertent mixing of eggs or larvae, progeny migration between tanks, or the chance mutation at microsatellite loci. No such presence was recorded in this family line.

According to Mendelian inheritance, the theoretical mating of two parents that are both heterozygous at two loci will produce 16 possible genotypes in equal proportions. In a total of 72 progeny we would therefore expect to see each of those genotypes an average of 4.5 times. In this example, five of the expected genotypes are not recorded at all. Of the 72 progeny examined, only five of them (< 7%) carry the 236 bp allele at *CmrHr* 2.30, when 36 would be expected (50%). The 236 bp allele does not appear to be deleterious to the survival of pure greenlip abalone as it is present in three of the eight male broodstock animals tested, one of them in the homozygous state. It appears obvious that there is some form of selection against sequence linked to this allele occurring in this family line. Reasons for this selection can only be hypothesised as it was not investigated further in this thesis. The 236 bp allele was also

seen in low frequencies in family 5, where the proposed greenlip male carried a single copy of the allele. One hypothesis is that this allele is linked to deleterious sequence in females, or in hybrid abalone. This would need to be confirmed by screening mature female abalone for the presence of this allele, and if found, conduct pair crosses which mate greenlip males and females with this allele to a number of blacklip males and females to produce hybrid offspring which should carry this allele. The selection may also involve polymorphisms in genes such as the lysin gene, which could then lead to non-random fertilization of *H. rubra* eggs by *H. laevigata* sperm.

Findings such as this one suggest that the application of molecular marker technologies to broodstock selection is an important step towards more reliable production of abalone in culture. If broodstock carrying such alleles can be avoided, then instances of poor survival such as that seen here can also be avoided. Alternatively, the use of more markers, with more alleles to investigate large numbers of family lines may lead to the development of loci which are positively linked to commercially important traits such as growth. Markers such as these are called quantitative trait loci (QTLs) and have been developed in more established farming and culture systems, and appear to have a bright future in the culture of abalone.

7.4.2.2 Families 4 and 5

Both of these family lines were considered to be single pair crosses between the broodstock listed in Tables 7-7 and 7-8. The results from genotyping the progeny suggest that this is not the case, with far too many alleles being present in the resultant progeny. Perhaps the most likely explanation for these discrepancies lies in the hatchery practices and control of activities prior to settlement of the larvae. In order to create pure family lines it is necessary to remove all possible causes of genetic contamination. Such contamination may arise as the result of equipment being shared between broodstock containers without sufficient sterilisation in between, such equipment include the mesh used for sieving excess sperm from fertilised eggs, as well as the containers used for the initial mixing of the gametes. In order to encourage egg production in females, some commercial hatcheries add small amounts of viable sperm to the water containing the female, therefore introducing more gametes to the spawning event. Contamination such as this may explain the addition of unwanted male gametes to subsequent progeny. Due to the size of abalone eggs (~250 microns diameter) it is unlikely that large scale contamination of a single pair cross could take place inadvertently, resulting in the unwanted female alleles that I observed.

In commercial enterprises it also common for successive production lots to be settled into the same raceway if stocking density appears low. This was not done intentionally in the research tanks, but must be considered as a possibility as I was not present during all settlement events.

Unfortunately, the limited variability of our two markers in the broodstock sampled makes it difficult to determine the likelihood of this event.

A further consideration is the rapid mutation of microsatellite loci during cross-species hybridisation to produce alleles that were not represented in either parental genotype. This seems unlikely given that no such mutation was observed within the progeny of family 2, although the detection of three previously unrecorded alleles in family 5 at *CmrHR* 2.30, at 339, 357 and 377 bp is unusual. All new alleles are larger than the allele range identified in the 12 blacklip female broodstock examined, but within the range of those recorded in wild caught blacklip abalone (Chapter 4). The larger 377 bp allele is also seen in the progeny of family 4, as is the foreign 291 bp allele at *CmrHr* 2.14, which is larger than any recorded allele at this locus in the greenlip male broodstock examined. Whilst this is not the most likely explanation for the results of this study, it is a possibility as microsatellite mutation rates as high as 0.05 per gamete have been found in humans (Jeffreys *et al.* 1988; Wolff *et al.* 1988), and non parental alleles have been found in hatchery reared progeny of the prawn, *Penaeus vannamei* at one microsatellite locus (Wolfus *et al.* 1997). A more likely explanation for the identification of these new alleles in both my study and the prawn study of Wolfus *et al.* (1997) however, is their presence in other broodstock that were not sampled. Only those animals thought to have contributed gametes to each family were sampled during this study, with many other animals not tested. For this reason, any future examinations of parentage in abalone culture would be well advised to sample all broodstock maintained on the site, thus removing one potential problem.

7.5 Conclusion

This study demonstrates the utility of highly polymorphic microsatellite markers as a means to compare genetic variation between wild and hatchery reared abalone. They reveal a decline in the number of alleles from wild to farm samples in both *H. rubra* and *H. midae*. There is a demonstrated change in allele frequency from the Tasmanian wild samples to the hatchery samples, resulting in more common alleles becoming rare, and rare alleles becoming the most common in some cohorts. With the potential for local adaptation of abalone stocks it is possible that such large changes in allele frequency could influence the fitness of hatchery stocks for continued culture or re-seeding efforts. No change was observed in overall heterozygosity in either of the species examined. The data show that these microsatellite loci are highly polymorphic in abalone, and are therefore a useful tool for pedigree analysis in genetic breeding programs. They also suggest that there are unanswered questions regarding mutation and inheritance in hybrid abalone. In pure species crosses however, microsatellites may provide a powerful tool for the genetic mapping of abalone species and the future development of quantitative trait loci for marker assisted selection programs.

Chapter 8 Identification of Southern Hemisphere abalone (*Haliotis*) species by PCR-RFLP analysis of mitochondrial DNA

8.1 Prelude

The completed protocol arising from this research is presented as the peer reviewed article of the same title that has been submitted to the *Journal of Shellfish Research* for publication. The article as submitted, and presented below, describes the process following conception. It describes the technical development, and statistical validation of the protocol for possible forensic applications.

As stated in the earlier declaration, the final protocol is the collaborative effort of Dr Neville Sweijd (Department of Zoology, University of Cape Town), Mr Jason Bartlett (CSIRO Marine Research), Dr Nick Elliott (CSIRO Marine Research) and myself. The following elucidates my role in the conception and development of this research, which began when the Tasmanian Police Service approached our laboratories with the need for the discrimination of abalone, ideally by location as well as between species.

8.2 Lysin gene

DNA database searching for abalone species identification highlighted the phylogenetic work of Lee and Vacquier (1995) and its subsequent use by Dr. Sweijd (then a PhD student) in South Africa. Dr Sweijd's work aimed to differentiate the South African abalone species, *Haliotis midae* and *H. spadiceae* (Sweijd *et al.* 1998) using a section of the lysin gene previously published by Lee and Vacquier (1995). Initially our goal was to extend this existing examination of the lysin gene to include other Southern Hemisphere species. In collaboration with Dr Sweijd, I examined the 125 bp fragment of the lysin gene amplified by the AUS1, AUS2 primer combination (Sweijd 1999) in 7 abalone species from Australia and South Africa (*H. midae, H. spadicaea, H. rubra, H. conicopora, H. roei, H. laevigata, H. scalaris*). These primers were developed by Dr Sweijd from the existing sperm lysin sequences published by Lee and Vacquier (1995), and designed using the OLIGO (Ver 3.4) program to amplify a small section of DNA which contained some fixed differences between species. It was necessary to target a small section of DNA to ensure that a product could still be amplified from degraded tissue such as that found in canned or dried abalone.

The published sperm lysin DNA sequence of approximately 125 bp was aligned in seven species by Dr Sweijd and myself, and searched for the presence of unique restriction sites. The expected fragment sizes after digestion of this region with each of five enzymes (ScrF I - CC'N,GG; Hha I - G,CG'C; Hae III - GG,'CC; Mae II - A'CG,T; Bal I - TGG',CCA) were predicted to separate all but one pair of species (Table 8-1). The unresolved pair being *H. laevigata* and *H. scalaris*. Using the AUS primer combination I was able to amplify a

product from only six of the seven species tested, no product could be amplified from *H. roei* DNA.

Restriction digestion of the six amplified products was performed with each of the five restriction enzymes in order to validate the restriction sites identified in the published sequences (Table 8-2). The *Bal* I restriction site identified in all but *H. conicopora* and *H. roei* sequences failed to cut in any of the species tested, and fragments from all species were cut only once by the *Mae* II enzyme. The disparity between sequence data and RFLP data may be explained by the miscalling of at least one base in those sequences, leading to the addition or removal of a predicted restriction site in some species. The enzyme activity was checked and shown to be intact by digestion of a Lambda DNA control.

Due to the detection of insufficient variation in this region for discrimination of *H. scalaris* from *H. laevigata* and *H. rubra* from *H. conicopora*, as well as the failure to amplify in *H. roei*, this region of the lysin gene was abandoned for subsequent protocol development.

Table 8-1 Predicted fragment sizes of an approximately 125 base pair fragment of the sperm lysin gene in 7 species. Restriction sites identified from published sequences (Lee and Vacquier 1995; After Sweijd 1999).

	Hha I	*ScrF* I	*Hae* III	*Mae* II	*Bal* I
H. midae	93, 32	125	102, 23	81, 44	102, 23
H. spadiceae	127	66, 61	104, 23	56, 44, 27	104, 23
H. laevigata	93, 32	75, 48	73, 27, 23	52, 44, 27	100, 23
H. scalaris	93, 32	75, 48	73, 27, 23	52, 44, 27	100, 23
H. rubra	93, 32	123	73, 27, 23	96, 27	101, 22
H. conicopora	93, 32	123	73, 27, 23	96, 27	123
H. roei	122	122	73, 27, 23	95, 27	122

Table 8-2 Observed fragment sizes after digestion of the AUS 1 and 2 primer combination amplified PCR product in 6 species. No product was amplified from *H. roei* DNA.

	Hha I	*ScrF* I	*Hae* III	*Mae* II	*Bal* I
H. midae	93, 32	125	102, 23	81, 44	125
H. spadiceae	127	66, 61	104, 23	100, 27	127
H. laevigata	93, 32	75, 48	73, 27, 23	96, 27	123
H. scalaris	93, 32	75, 48	73, 27, 23	96, 27	123
H. rubra	93, 32	123	73, 27, 23	96, 27	123
H. conicopora	93, 32	123	73, 27, 23	96, 27	123
H. roei	-	-	-	-	-

I then attempted to amplify the larger, intron containing lysin fragment using the HGEN (Sweijd 1999) primer pair (Table 8-3). It was hypothesised that obtaining sequence for this large section of DNA would assist in our search for a region of the gene containing sufficient

variation to discriminate all seven species. This approach was foiled by the failure to amplify a product from *H. scalaris* and *H. laevigata*. This failure was thought to be due to the large size of the intron (~4 Kbp) in these two species. To detect a large DNA insertion event, a combination of *Taq* DNA polymerase and *Pfu* polymerase was used in a long PCR protocol designed to amplify products of up to 4 Kbp in size. This protocol uses a two-step PCR cycle (denaturation at 94° C, and annealing / extension at 62° C), and the *Pfu* polymerase has a proofreading capability that enables the more accurate production of longer DNA fragments. Despite these attempts, no product could be amplified for *H. laevigata*. A product of approximately 3 800 bp was amplified from *H. scalaris* using these methods, but subsequent sequencing of this product was problematic.

Table 8-3 Details and sequences of PCR primers used in the development of the abalone species identification protocol. R = A + G; W = T + A; I = Inosine; Y = C + T.

Marker	PCR primer combination	Sequence 5' - 3'	Product Size (~bp)	primer Source
Mitochondrial	HMND1d c16sar	ACT ARC TCR GAT TCT CCT TCW GCA A CCT CGC CTG TTT AGC AAA AAC AT	1600	Sweijd 1999
	HALCO1A HALCO1B	ATT GTA ACA GCT CAT GCT TTC GTT TGC TCC AGC AAG TAC TGG GAG	420	Sweijd 1999
	HALCO1-NG1 HALCO1-NG2	CIG ACA TRG CIT TYC CIC GAC T CCG GCT ARG TGI AGI GAR AAA AT	193	new
	HALCO2C HALCO2D	TTA TRT TAA GAT CTC TCA CAT CCC G TRA AAC TTG TTT GGT TAA GGC G	420	Sweijd 1999
	HALCO2A HALCO2B	ATC TGA ACC ATT CTC CCA GCC CCT TAA AGT CYG AGT ATT CGT AGC C	157	new
Nuclear lysin gene	HGEN1 HGEN2	GGT CAG GAG AAA CTT GAT CCC ACT ATY GWA CAA TGT TTA CGA GTT AAA TAG A	650	Sweijd 1999
	AUS1 AUS2	TGG AAC CAA TGC ACA ACC GTG AAT AYA TCG GGT GAA AAA TCT GG	125	Sweijd 1999

8.3 Mitochondrial DNA

Because of the difficulties encountered during my attempts to extend the lysin gene protocol, we began to investigate regions of mitochondrial DNA. Four regions of the mitochondrial DNA genome were identified for which there was either sequence data or working primers available. The areas examined for fixed interspecific variation were the 16S rRNA (16S) gene,

NADH dehydrogenase Subunit I (ND I) gene and the Cytochrome Oxidase I (CO I) and II (CO II) genes. At this stage the number of species to be included for differentiation was increased to 11 in order to include all the southern hemisphere species of major commercial interest. The species added to the test were three species from New Zealand: *H. iris*, *H. australis* and *H. virginea*, and the tropical abalone *Haliotis asinina* from northern Australia.

16S rRNA sequence was available for *H. discus hannai* (Jiang *et al.* 1995). The HMND1d, c16sar primer combination (Table 8-3) was used to amplify approximately 1600 bp in ten species. Due to its size, it was possible to obtain sequence in only one direction at either end of the product. Good quality sequence was obtained and aligned for ~230 bp of the 16S rRNA gene (Figure 8-1). No product was amplified from *H. laevigata*. Aligned sequence was examined for species-specific restriction sites.

ND I sequence resulted from sequencing the HMND1d, c16sar product from above with the HMND1d primer (Table 8-3). Good quality sequence was obtained and aligned for a region of approximately 190 bp of the ND1 gene in 10 species (Figure 8-2). Aligned sequence was examined for species-specific restriction sites.

Both the 16S rRNA and the ND I regions of the mitochondrial genome contained interspecific polymorphisms that could have been utilised to discriminate these species. However, the failure to produce an amplification product from *H. laevigata* in this region, and the rapid success achieved for all species using the Cytochrome Oxidase genes described below, meant that this work was not followed through to completion.

CO I sequence was available for 11 abalone species (*H. cyclobates, H. iris, H. rubra, H. corrugata, H. fulgens, H. midae, H. cracherodii, H. discus hannai, H. wallensis, H. kamtschatkana, H. sorenseni*) from both hemispheres (Metz *et al.* 1998). I used the HALCO1A, HALCO1B primer combination (Table 8-3) to amplify a 420 bp region of the CO I gene in five Australian (*H. roei, H. asinina, H. laevigata, H. scalaris, H. conicopora*), one South African (*H. spadicea*) and one New Zealand (*H. australis*) species that were not sequenced by Metz *et al.* (1998). Sequence for all 18 species available was then aligned and I designed a set of internal generic primers named HALCO1-NG1 and HALCO1-NG2 (Table 8-3) to amplify a product of ~ 193 bp from all 11 southern-hemisphere species examined. This

seqNav-ALIGNED 8/9/01 12:25 PM

```
                10        20        30        40        50
discus hannai ATGAGGAGTC GGACCTGCCC GGTGAC*TAC GGGTTAAACG GCC-GCGGTA
midae         ---------- ---------- ------C--- ---------- ----------
scalaris      ---------- ---------- ------C--A ---------- ----------
roei          ---G------ ---------- A-----C--A ---------- ---C------
asinina       --A------- ---------- ------C--- ---------- ----------
spadicea      ---------- ---------- ------C--- ---------- ----------
iris          --A------- ---------- ------C--- ---------- ----------
virginea      --A------- ---------- ------C--- ---------- ---C------
australis     --A------- ---------- ------C--- ---------- ----------
conicopora    ---G------ ---------- ------C--A ---------- ----------
rubra         ---G------ ---------- ------C--A ---------- ---C------

                60        70        80        90       100
discus hannai CACTGACC*G TGCAAAGGTA GCACAATCAC TTGCCCTTTA ATTAGAGGCT
midae         --------*- ---------- ---------- -----T---- ---G------
scalaris      --------*- ---------- ---------- -----T---- ---G------
roei          --------*- ---------- ----------T -----T---- ---G------
asinina       --------*- ---------- ---------- -----T---- ---G------
spadicea      --------*- ---------- ---------- -----T---- ---G------
iris          --------*- ---------- ---------- -----T---- ---G------
virginea      --------*- ---------- ---------- -----T---- ---G------
australis     --------*- ---------- ---------- -----T---- ---G------
conicopora    --------*- ---------- ---------- -----T---- ---G------
rubra         --------C- ---------- ---------- -----T---- ---G------

               110       120       130       140       150
discus hannai GGTATGAATG GTTTGACGAG GGTTGAGCTG TCTCTTTTGG AATAATTTAA
midae         ---------- ---------- --C------- ------C--- ----*-----
scalaris      ---------- ---------- --C------- ------C--- ----*-----
roei          ---------- ---------- --C------- ------C--- ----*-----
asinina       ---------- ---------- --C------- -------C-- ----*-----
spadicea      ---------- ---------- --C------- ------C--- ----*-----
iris          ---------- ---------- --C------- -----CC-T- ----*-----
virginea      ---------- ---------- ---------- -----CC-C- ----*-----
australis     ---------- ---------- --C------- ------C--A ----*-----
conicopora    ---------- ---------- --C------- ------C--- ----*-----
rubra         ---------- ---------- --C------- ------C--- ----*-----

               160       170       180       190       200
discus hannai AAATTAACTT CTAGGTGAAA AGGCCTAGAT TGAGCTGAGG GAC*GAGAAG
midae         ---------- ---------- ----T----- -T-------- ---*------
scalaris      ---------- ---------- ----T--A-- -T-------- ---C------
roei          ---------- ---------- ----T----- -T-------- ---*------
asinina       ---------- ---------- ---------- ---------- ---*------
spadicea      ---------- ---------- ----T--T-A -T-A------ ---*C-----
iris          ---------- ---------- ---------- -A-------- ---*------
virginea      ---------- ---A----G- ----T----- -A-------- ---*------
australis     ---------- ---------- ----T----- ---------- ---*C-----
conicopora    ---------- ---A------ ----T----- -T-------- ---*------
rubra         ---------- ---A------ ----T----- -T-------- ---*------

               210       220       230       240       250
discus hannai ACCCTGTTGA GCTTTAGTGT TGGGTAGAGA G
midae         ---------- ---------- G-----A--G T
scalaris      ---------- ---------- G-AA--A-AG -
roei          ---------- ---------- G--A--A-AG -
asinina       ---------- ---------- --AA--A--G T
spadicea      ---------- ---------- G-----A--- -
iris          ---------- ---------- G-A--G---* *
virginea      ---------- -------C-- G--A--A-*G T
australis     ---------- ---------- G-AA-GA--G T
conicopora    ----C----- ---------- G-----A-AG -
rubra         ----C----- ---------- G-----A-AG -
```

Figure 8-1 Alignment of 16S rRNA sequence for 10 abalone species from the southern-hemisphere. All sequence is aligned to the published sequence of *H. discus hannai* (Jiang *et al.* 1995). - signifies a conserved base position, * signifies a deletion of a base in that species.

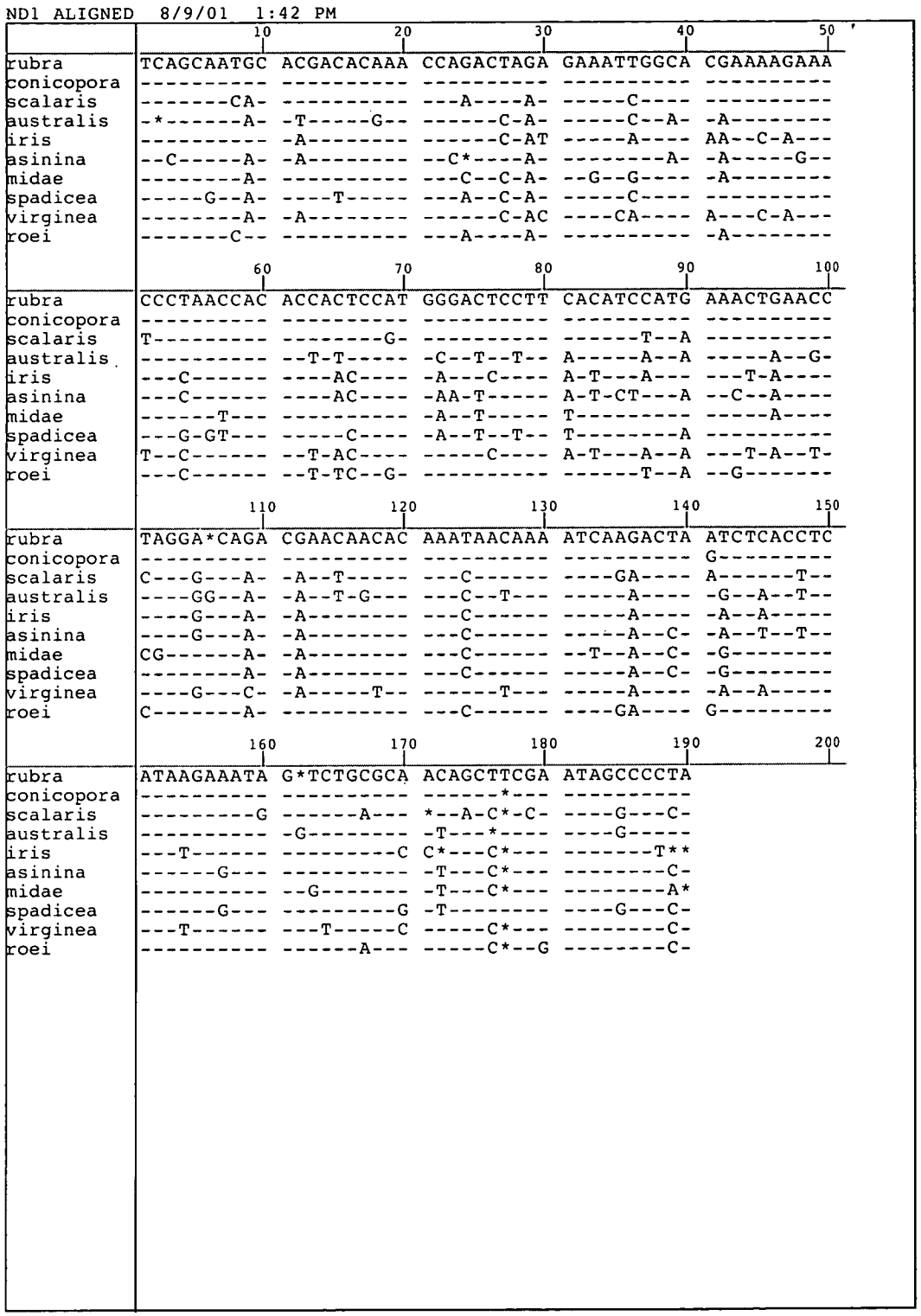

Figure 8-2 Alignment of NADH dehydrogenase Sub-unit 1 sequence for 10 abalone species from the southern-hemisphere. All sequence is aligned to the nucleotide sequence of *H. rubra.* - signifies a conserved base position, * signifies a deletion of a base in that species.

region of DNA provided the most conserved region for primer design and the required level of variation for the discrimination for the majority of southern-hemisphere species, and was therefore developed further to produce the protocol described in the accompanying manuscript (Elliott *et al.* submitted).

CO II gene sequence was available for nine of the abalone species of interest to us (Sandie Degnan, unpublished data). The HALCO2C, HALCO2D primer combination (Table 8-3) was used to amplify a product of approximately 420 bp in the remaining species, *H. conicopora* and *H. spadiceae*. All sequences were aligned using Sequence Navigator (Ver. 1.0.1), and I designed a set of internal generic primers named HALCO2A and HALCO2B (Table 8-3) using OLIGO (Ver 5.0). These primers were designed to amplify a product of ~ 157 bp in *H. laevigata* and *H. scalaris*, as these species could not be separated by variation at the CO I gene. Further testing on other species produced an amplification product in all species except *H. iris* leading to the development of this region as a component of the final protocol described in the manuscript (Elliott *et al.* submitted).

The accompanying manuscript (Elliott *et al.* submitted) details the methods of primer design, PCR amplification and RFLP analysis. The enzymes selected for the PCR-RFLP analysis were chosen after the alignment of all available sequences using the DAPSA program (Harley 1996). Restriction site differences were identified using DAPSA, and all polymorphisms were tested by restriction digestion of the amplified fragment in at least two individuals of each species (extra individuals were examined by Mr Bartlett as outlined in the manuscript).

8.4 Haliotis rubra / Haliotis conicopora

Sequence data revealed little basis for the recognition of *H. rubra* and *H. conicopora* as separate species. All available sequence data for these two species have been compiled in Figure 8-3. These data represent over 1800 bp of mitochondrial and nuclear DNA sequence for these two species which differ at only 10 sites, a divergence of only 0.53%. This compares to a sequence divergence of 8.27% in these same regions between *H. rubra* and another Australian species *H. scalaris* (unpublished data). Metz *et al.* (1998) suggest that abalone species can have as much as 0.4% nucleotide divergence in the CO I gene, with between 3.7 and 64.9% divergence noted within that gene between species. An examination of the Lysin gene by Vacquier *et al.* (1997) suggests that abalone species may differ at between 4.5 and 48.6% of their nucleotide positions, whilst less than 1% of those sites will vary between individuals of the same species. The inability of all approaches to discriminate these "species" is therefore not suprising, and may also be unnecessary in a species identification context.

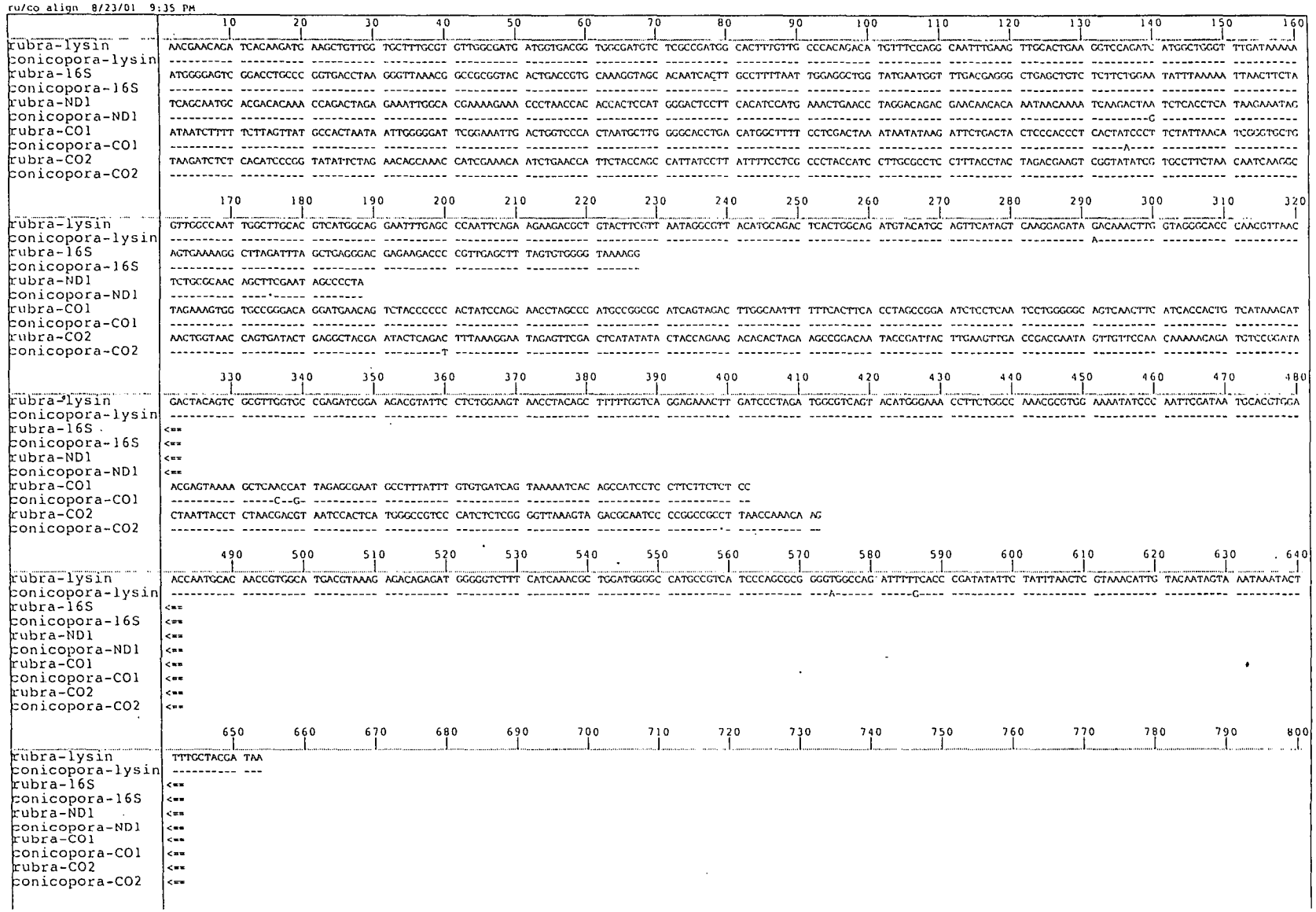

Figure 8-3 Alignment of *H. rubra* and *H. conicopora* over 1800 bp of sequence from 1 nuclear and 4 mitochondrial regions. Lysin sequence from Lee and Vacquier (1995); H. rubra CO II sequence from Sandie Degnan (unpublished); H. rubra CO II sequence from Metz et al (1998); All other sequences obtained at CSIRO Marine research. * signifies a base deletion in that sequence.

In Chapter 3 of this thesis I present the results of a study of the conservation of 22 *Haliotis rubra* microsatellite loci in 12 other species of abalone. Fifteen of the loci amplified a product of the expected size range in *Haliotis conicopora*, at least three more than in any other species tested. It is intended that the multiplex PCR reactions outlined in Chapter 4 of this thesis be used to examine samples of *H. rubra* from South Australia, and *H. conicopora* from Western Australia. This examination will provide more information in the debate of the *H. rubra / H. conicopora* specific status.

8.5 Elliott et al. Submitted

Identification of Southern Hemisphere abalone (*Haliotis*) species by PCR-RFLP analysis of mitochondrial DNA

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ABSTRACT

Illegal fishing and species-substitution of abalone (genus *Haliotis*), a highly valuable marine gastropod, are of worldwide concern. A mitochondrial DNA PCR-RFLP analysis of fragments of the cytochrome oxidase I (mtCOI) and II (mtCOII) genes was developed for the identification of 11 Southern Hemisphere species of abalone. These included five temperate and one tropical species from Australian waters, three temperate species from New Zealand and two temperate species from South Africa. All species, with the exception of the *Haliotis rubra*/ *H. conicopora* complex, can be unequivocally identified using the combined profiles from four individual restriction enzyme digests (*DdeI*, *HhaI*, *HinFI* and *HpaII*) on a 193 bp fragment of mtCOI. Six species each displayed a unique profile for a single restriction enzyme. A 159 bp fragment of mtCOII allowed individual identification of six of the species using the combined profiles from five individual restriction enzyme digests (*DdeI*, *EcoRV*, *HhaI*, *HpaII*, and *RsaI*). These primers failed to amplify in *H. iris*. Again *H. rubra* and *H. conicopora* could not be separated, and neither could *H. australis* and *H. spadicea*. No DNA sequence variation in either fragment was observed between *H. rubra* and *H. conicopora*; the latter may be a subspecies of *H. rubra*. The use of both fragments and a minimum of two restriction enzymes is recommended for species differentiation. DNA was successfully extracted, PCR amplified and identified from canned tissue and mucous samples of *H. rubra*. A conformational mutation in the mtCOI fragment was observed in *H. midae*, but in no other species nor in the mtCOII fragment.

KEY WORDS: Haliotis, abalone, mitochondrial DNA, identification

RUNNING TITLE: Southern Hemisphere abalone identification

INTRODUCTION

Abalone, genus *Haliotis* Linnaeus, are a highly valuable commercial marine univalve mollusc. There are over 55 recognized species worldwide (Geiger 1998), of which nearly half are exploited by commercial or recreational divers. Abalone generally inhabit rocky reefs to depths of 65 m, but are more usually found in shallower waters to 30 m. The foot muscle of abalone attracts high prices in Asian markets, with species differential. Once removed from the shell and trimmed of distinguishing mantle tissue, it is very difficult to differentiate the commercial product of one species from another. The high price, market demand, ease of harvest and similarity of processed product between species makes abalone very suitable targets for illegal marketing and both highly organised and small scale poaching.

Abalone poaching and species-substitution of abalone products is of concern to many countries, including the USA (Daniels and Floren 1998), Mexico (Ponce-Díaz et al. 1998), South Africa (Sweijd et al. 1998) and Australia. The value of the illegal trade is difficult to quantify. Conservative estimates in Australia alone are over $US25M annually. The legal Australian abalone fisheries, dominated by *Haliotis rubra,* account for about half the annual world abalone harvest of ca. 10 500 mt (FAO 2000) and is worth around $US80M per year. The South African abalone fishery *(H. midae* ca. 500 mt/yr) is worth approximately $US15M with legal sales of confiscated (poached) abalone from just one area fetching over $US1m (Sweijd et al.1998). In New Zealand the main commercial species is *H. iris* and the illegal harvest is estimated at about 33% of the annual commercial catch of 1 300 mt (Roberts et al. 1999). The high but unknown level of illegal harvesting of abalone creates major problems for fishery managers endeavoring to maintain viable and economic fisheries.

Whilst *H. rubra* is the dominant commercial species within temperate Australian waters, both *H. laevigata* and *H. roei* are subject to significant levels of commercial fishing under independent quota systems, and a *H. scalaris* fishery is under consideration. A problem for fisheries enforcement is the overlapping ranges of these and non-commercial species. Such species richness is common with abalone (Geiger 1999), and once the shell and mantle have been removed identification of the commercial product is obscure. The need exists for a definitive means for identifying tissue and by-products (e.g. mucous in instances of suspected poaching when tissue has been disposed) of individual abalone species.

Identification of plant and animal species when morphological characters have been removed is possible using either protein or DNA-based methods (Palumbi and Cipriano 1998, Toro 1998, Johannesson and Stenlid 1999, Hare et al. 2000, Sweijd et al. 2000). The protein based methods are very dependent on tissue quality, generally requiring fresh or frozen material. Often identification for commercial needs may require analysis of processed (dried or canned) tissue or degraded tissue. DNA-based methods are relatively independent of tissue quality, and those that rely on amplification of small DNA fragments are less likely to be affected by degradation (Mackie et al. 1999). A number of techniques are available for species identification including: random amplification of polymorphic DNA (RAPD) (Martinez and

Malmheden Yman 1998), restriction fragment length polymorphism (RFLP) analysis (Innes et al. 1998, Wolf et al. 2000), direct DNA sequencing (Quinteiro et al. 1998) and single-stranded conformation polymorphism (SSCP) (Mackie et al. 1999) of PCR (polymerase chain reaction) amplified fragments.

A PCR-RFLP analysis of a 1,300 base-pair (bp) fragment of the nuclear lysin gene was devised for identification of two South African abalone species, *Haliotis midae* and *H. spadicea* (Sweijd et al. 1998). Generic PCR primers that amplify across the intron differentiated between species based on the size of the intron. Preliminary analyses found that the size of the lysin intron varied greatly between other *Haliotis* species (generally 500 to 1 100 bp), but the intron in the Australian greenlip abalone *H. laevigata* was over 4 000 bp (unpublished data). Products of such size are not ideal for species identification tests with the likelihood of unreliable PCR products due to tissue and DNA degradation.

To differentiate between the more common Southern Hemisphere abalone species a PCR-RFLP method was developed using short fragments (less than 200 bp) of the mitochondrial DNA (mtDNA) molecule. To satisfy potential legal scenarios in Australia and South Africa, 11 species were included. Within species variation and potential non-*Haliotis* amplification of our designed primers were examined in addition to testing the primers with canned abalone tissue and abalone mucous samples.

MATERIALS AND METHODS

Sample collection and DNA extraction

Whole individuals (live or frozen) or alcohol preserved tissues were obtained for 11 purported *Haliotis* species:

Haliotis asinina Linnaeus	Queensland, Australia	(30 individuals)
Haliotis australis Gmelin	New Zealand	(10)
Haliotis conicopora Péron	Western Australia, Australia	(11)
Haliotis iris Gmelin	New Zealand	(10)
Haliotis laevigata Donovan	Tasmania & Victoria, Australia	(62)
Haliotis midae Linnaeus	South Africa	(10)
Haliotis roei Gray	Western Australia, Australia	(10)
Haliotis rubra Leach	Tasmania, Victoria & New South Wales, Australia	(50)
Haliotis scalaris (Leach)	Tasmania, Australia	(20)
Haliotis spadicea Donovan	South Africa	(10)
Haliotis virginea Gmelin	New Zealand	(10)

Total genomic DNA was extracted from ca. 25 mg of foot muscle or gill tissues using a modified CTAB (hexadecyltrimethylammonium bromide) protocol (Grewe et al. 1993). Tissue was incubated overnight at 50°C instead of 30 to 60 min at 60°C.

To verify the use of our PCR primers on processed product, DNA was extracted from commercially canned *H. rubra*. Approximately 0.5 g tissue was digested for 30 min at 65°C in 5 mL digestion buffer (100 mM Tris, 50 mM EDTA, 400 mM NaCl, 1% SDS). 50 µL proteinase K (10 mg/mL) was then added and the solution was incubated overnight at 55°C. 150 µL NaCl (5 M) and 520 µL of 10%

CTAB were added and the solution incubated at 65°C for 1 hr with regular mixing. Samples were then extracted once with equal volumes of chloroform/isoamyl alcohol (24:1) and precipitated with 2 volumes of 100% ethanol. Precipitated DNA was washed twice with 70% ethanol, once with 100% ethanol, air-dried and re-suspended in 200 µL TE.

PCR amplification was also tested using DNA extracted from *H. rubra* mucous. Two mucous samples were obtained by placing individual freshly captured *H. rubra* in separate plastic bags for approximately 2 h, removing the abalone and placing the bag and fluid contents at 4°C. Tissue samples were taken from the individual abalone as positive controls for DNA extraction. DNA was extracted from mucous swabs taken from the sides of the bags and from the control tissue samples using the modified CTAB protocol described above. In addition, a 600 µL sample of fluid (mixture of seawater and mucous) from the bottom of each plastic bag was taken, incubated overnight in 20 µL proteinase K (10 mg/mL) and 5% SDS, and then genomic DNA extracted using the same modified CTAB protocol.

Genus specificity of the PCR amplification was tested on total genomic DNA extracts (using above CTAB protocol) from a variety of marine organisms. These consisted of an alga (unidentified red alga), an anemone (unidentified), a crustacean (Antarctic krill *Euphausia superba*), molluscs (unidentified chiton and Pacific oyster *Crassostrea gigas*) and teleosts (bigeye tuna *Thunnus obesus*, southern bluefin tuna *T. maccoyii*, pink ling *Genypterus blacodes*, Patagonian toothfish *Dissostichus eleginoides*, school shark *Galeorhinus galeus* and gummy shark *Musteleus antarcticus*).

PCR primers and amplification

Generic PCR primers were designed for the mitochondrial cytochrome *c* oxidase subunit I gene (mtCOI) by alignment of either our own unpublished or published *Haliotis* sequences (Metz et al. 1998). DNA sequences used for the design of the mitochondrial cytochrome *c* oxidase subunit II gene (mtCOII) were either our own or other unpublished sequences (Sandy Degnan, University of Queensland).

The primers designed to amplify a 193 bp fragment of the mtCOI gene were designated HALCO1-NG1 (5'-CIGACATRGCITTYCCICGACT-3') and HALCO1-NG2 (5'- CCGGCTARGTGIAGIGARAAAAT-3'). Those designed for a 159 bp fragment of the mtCOII gene were designated HALCO2GENA (5'-CAATYTGAACYATTCTMCCAGC-3') and HALCO2GENB (5'-CCTTAAARTCTGAGTATTCGTAGCC-3'). (Degenerate nucleotide IUB codes: I, Inosine = A, C, G or T; M, aMino = A or C; R, puRine = A or G; Y, pyrimidine = C or T).

PCR reactions consisted of 50 to 100 ng of total genomic DNA, 2.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmoles of each primer, and 0.55 U *Taq* DNA polymerase (Biotech) in a buffer supplied by the manufacturer. PCR amplifications were carried out in a 50 µL final volume using a Perkin Elmer GeneAmp® System 9600 with hotlid. The cycling parameters were as follows: denaturation at 95°C for 3 min, 10 initial amplification cycles (94°C for 30 s, 60–55°C for 30 s, 72°C for 1 min, with a decrease in the annealing temperature of 0.5°C per cycle), a further 25 amplification cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) and final extension

at 72°C for 5 min. Negative controls, without DNA template, were prepared for each series of amplifications to exclude the possibility that PCR reagents and buffers were contaminated with template DNA. Amplification products were examined by electrophoresis through a 2% agarose gel (GIBCOBRL) made up in 1 X TBE. Gels were stained in ethidium bromide at a concentration of 0.5 μg/mL and visualised under UV light. A 100 bp ladder (GIBCOBRL) was run concurrently to facilitate sizing of amplification products.

DNA Sequencing

PCR products were sequenced to confirm variation in restriction fragments and sizes, and to improve PCR primer design. PCR products were purified using Wizard™ PCR purification columns (Promega) according to manufacturers instructions, and sequenced using an ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Cycle sequencing reactions were electrophoresed on an ABI377 automated DNA sequencer (Perkin Elmer) and analyzed using ABI Prism™ Sequencing Analysis Version 3.3 (Perkin Elmer).

RFLP analysis

For each individual of the 11 species, four separate restriction digestions of the mtCOI fragment were performed using the four enzymes *DdeI, HhaI, HinFI* and *HpaII* (New England Biolabs, Genesearch). For the mtCOII fragments five separate restriction digestions were performed for each species individual using the enzymes *DdeI, EcoRV, HhaI, HpaII* and *RsaI* (New England Biolabs, Genesearch). Restriction digestions were carried out in a 15 μL total volume consisting of 5 μL of PCR product, 1.5 μL digestion buffer supplied by the manufacturer, 0.5 μL enzyme, and 8 μL dd$H_2$0 for all enzymes except *HhaI*. Digestions for *HhaI* were carried out in a 15 μL total volume consisting of 5 μL of PCR product, 1.5 μL digestion buffer supplied by the manufacturer, 0.5 μL enzyme, 1.5 μL 10X Bovine serum albumin (BSA) and 6.5 μL dd$H_2$0.

Mitochondrial haplotypes were scored by electrophoresis of 10 μL of digested PCR product in a 3% agarose gel made up in 1 X TBE at 100V for 3 hr, stained in ethidium bromide (0.5μg/mL) and visualised under UV light. Electrophoresis of restriction digestions was also performed on 12% polyacrylamide (Austral Scientific) gels made up in 1 X TBE and run for 2 hr at 100 V.

RESULTS

DNA extraction and PCR amplification

DNA extractions from fresh, alcohol preserved and canned tissue, resulted in high yields of high molecular weight total genomic DNA. Amplification of these extracts consistently produced high quality PCR products.

Extractions from mucous scrapings and fluid samples from plastic bags produced a small amount of high molecular weight genomic DNA. PCR amplification of these extracts failed at times to yield a product when undiluted, however when diluted 10 fold, produced a strong PCR product in all samples (Fig. 1)

PCR amplification of non-*Haliotis* DNA with the designed primers was observed only in the tuna samples. Both tuna species amplified (160 bp fragment)

with the mtCOI primers. Sequencing of the tuna mtCOI products confirmed that the observed product was not contamination from abalone DNA. While nucleotide differences and RFLP cut site differences existed to separate these teleost products from abalone products, high levels of nucleotide sequence conservation suggests that the amplified product was part of the tuna COI gene.

Restriction digests mtCOI

The expected 193 bp fragment was generated in each abalone species following PCR amplification with the HALCO1-NG1/HALCO1-NG2 primers. Comparison of the DNA sequences indicated suitable restriction sites for discrimination between species using four restriction enzymes (Fig. 2).

Intraspecies restriction digest polymorphisms were observed in four species, but in each case for a single individual for only one enzyme (Table 1). Two of the observed polymorphisms were the result of a loss of a restriction site and two the result of a gain. All individuals showing a different restriction fragment profile for the species were sequenced to confirm the profile. All other digestions returned a single restriction pattern in all individuals examined for each species.

At this 193 bp fragment, six of the eleven species had a unique species-specific restriction pattern for at least one enzyme, and so could be individually identified (Table 1). With the exception of the *H. rubra* and *H. conicopora* pairing, all species are discernible from each other using the four restriction enzymes, regardless of all but one observed polymorphism. The exception polymorphism was a single *H. rubra* individual that had gained a *DdeI* cut site, and therefore had a profile similar to *H. scalaris*. The restriction profiles for the canned tissue, mucous and fluid samples all matched that expected for *H. rubra.*

One purported *H. scalaris* individual returned a different profile at three enzymes to all other *H. scalaris* individuals. This particular individual displayed the expected cut pattern for *H. laevigata* for all four enzymes; three of which are diagnostic between the two species for all other specimens analyzed. Laboratory contamination was ruled out and this result confirmed with repeated tissue sampling, DNA extraction and PCR amplification for this one individual.

The observed fragment lengths produced in this study were all examined on agarose and (non-denaturing) polyacrylamide gels and confirmed by sequence analysis. A fragment mobility change was observed in the mtCOI fragment for *H.midae* when run on a polyacrylamide gel (Fig. 3). This assumed conformation-induced mutation was only observed in *H. midae*.

Restriction digests mtCOII

The expected 159 bp fragment was generated in each abalone species following PCR amplification with the HALCO1GENA/HALCO2GENB primers, except *H. iris* that failed to amplify for all ten individuals examined. Comparison of the DNA sequences for the other species indicated suitable restriction sites for discrimination between species using five restriction enzymes (Fig. 4).

All restriction digestions for the five enzymes resulted in a single restriction pattern for each species, except for two enzymes for *H. rubra* (Table 2). The two

polymorphisms were each observed in two different individuals, all were sequenced to confirm the observed RFLP. None of these four individuals were responsible for the polymorphisms observed at the mtCOI fragment, and the individual *H. rubra* with a mtCOI profile similar to *H. scalaris* was clearly identified as *H. rubra* at this fragment. The restriction profiles for the canned tissue, mucous and fluid samples all matched that expected for *H. rubra.*

As with the mtCOI RFLP analysis, an unusual species profile was observed for three enzymes with a single *H. scalaris* individual (the same individual), and again all three profiles match that recorded for *H. laevigata.* Sequence data showed a 100% similarity to *H. laevigata* across the 159 bp fragment, while three other *H. scalaris* samples each differed at 9 nucleotides from the *H. laevigata* sequence.

At this 159 bp fragment, three of the ten species (excluding *H. iris* that did not amplify) had a unique restriction pattern for the enzyme *DdeI* and so could be individually identified (Table 2). In addition to the *H. rubra/H. conicopora* complex it was not possible to separate *H. australis* and *H. spadicea* using the five enzymes on this fragment. All remaining species combinations were separable from each other using one to five of the enzymes (Table 2).

Fragment mobilities on polyacrylamide gels were all consistent with known fragment lengths; no conformation induced mutations were observed in the mtCOII fragment.

DISCUSSION

The ability to identify abalone species from tissue samples and/or mucous is important to the continued survival of significant abalone fisheries. The tests described in this paper will provide one more tool in the fight against illegal fishing, which has the potential, along with commercial over-fishing and environmental variables (Davis et al. 1998, Shepherd et al. 1998), to lead to the decline and collapse of fisheries. The methods are straightforward and suitable for use in any laboratory with basic DNA analytical equipment. The PCR-RFLP tests utilize short DNA fragments that can be amplified from processed products and slightly degraded material, and therefore are of potential forensic use.

Care has been taken in this study to include examination of intraspecies variation as well as possible non-*Haliotis* amplification with our PCR primers. Samples from different geographic locations were examined for the two main Australian commercial species (*H. rubra*, five locations and *H. laevigata*, three locations). While not exhaustive, the results suggest that what limited intraspecies variation exists can be accounted for using the two fragments and multiple restriction enzymes. Both PCR primer sets devised for the test are relatively degenerate and so cross genus amplification was not unexpected. However, of the groups we have examined only DNA from the tunas (*Thunnus* spp.) amplified, and it was possible to easily differentiate these from *Haliotis* species.

The restriction patterns produced by *DdeI* for the mtCOII fragment would discriminate three of the species, while five other species would be differentiated by a single restriction pattern at the mtCOI fragment. Such species-specific patterns are useful, however as rare polymorphisms may exist it would be wise to confirm

identification with multiple enzymes and/or both short fragments. None of the rare polymorphisms observed occurred in more than a single individual, and no individual displayed more than one variation. With the exception of the *H. rubra/H. conicopora* pairing, all other combinations of the eleven abalone species can be differentiated from each other using two or more of the restriction profiles shown in this study. We therefore recommend using both fragments and at least two of the restriction enzymes included here to differentiate species.

The ability of our test to differentiate between species was inadvertently put to the test during the intraspecies examinations. Of 20 purported *H. scalaris* individuals, one was found to display a different restriction profile at six of the nine profiles examined. The combined profile of this individual matched completely the expected profile for *H. laevigata*; and was confirmed by DNA sequence analyses. Although occupying different microhabitats, these two species have overlapping distributions and co-occur in the same area (Shepherd 1973). Shell and mantle morphology did not separate the aberrant individual from other *H. scalaris* individuals. This individual is either a *H. laevigata* and morphological characters between the two species are more plastic than currently recognized, or it is a hybrid between the two species.

Naturally occurring hybrids between abalone species with overlapping ranges, although relatively rare, have been reported (e.g. Talmadge 1977, Sasaki et al. 1980, Arai et al. 1982, Messier and Stewart 1994). The two Australian species *H. rubra* and *H. laevigata*, also show evidence of backcrossing and introgression (Brown 1995). There are no records of hybrids between *H. laevigata* and *H. scalaris*, but *H. laevigata* is more closely related to *H. scalaris* than to *H. rubra* (Brown and Murray 1992), and hybrids would not be unexpected. Allozyme analysis of the aberrant individual could not confirm nor refute its putative hybrid status as there are no known diagnostic loci between the two species (Brown 1991).

The possible existence of hybrids, albeit at low frequencies, does not minimize the validity of our mtDNA-based test for abalone. However, the possibility of hybridization and backcrossing between species does question the legal 'species identity' of an individual. If hybrids were infertile and only F1 hybrids were possible, then a single diagnostic nuclear DNA marker would confirm the individual as a hybrid, and the mtDNA marker would confirm the maternal species. Such individuals could be legally classed as hybrids. However at least some abalone hybrids appear to be fertile and backcrossing occurs (Brown 1995), and identifying the 'nuclear lineage' of a potential backcross offspring would require multiple nuclear DNA markers. Even then it could never be proved that an individual was not the offspring of a backcrossing event, except based on probability. A suite of nuclear DNA markers could never disprove a claim of backcrossing, although making it improbable. On the other hand using a mtDNA-based test, the maternal lineage of the individual can always be validated. We suggest that for legal purposes where hybrid backcrossing may exist between abalone species that the genetic 'species identity' of an individual be classified as its maternal lineage, which can be confirmed from its mtDNA. Hybrid individuals (those with mtDNA of one species and nuclear DNA wholly or partly of another species) while biologically acknowledged should not be legally recognized as the existence of backcross hybrids can not be disproved except by probability based on a large number of diagnostic nuclear DNA markers. Mitochondrial DNA in

abalone as in most organisms appears to be only maternally inherited (Conod 2000). The aberrant individual in our study therefore is classed as *H. laevigata*.

The advantage of the tests described here to previous studies (Sweijd et al. 1998) for abalone is the smaller size of the DNA fragment, an advantage when examining processed or slightly degraded material (Mackie et al. 1999). The lysin gene protocol described by Sweijd et al. (1998) did aim for fragments less than 300 bp, but the presence of an intron increased this at least three times, and for *H. laevigata* by about ten fold (unpublished data). The authors did however successfully use PCR primers for a smaller 146 bp fragment to discriminate between canned *H. midae* and *H. rubra* products.

PCR inhibition was observed when testing our primers on the mucous samples of *H. rubra*. Dilution (10 fold) to a lower concentration did not have the same inhibitory effect. Similar PCR inhibition due to high levels of polysaccharides is common in plant tissue extracts (Fang et al. 1992), and inhibition due to mucopolysaccharides in the abalone mucous may have caused the observed PCR failure.

No DNA sequence variation was observed between *H. rubra* and *H. conicopora* in either short mtDNA fragment examined in this study. In an assessment of all Recent taxa in the family Haliotidae, Geiger (1998) concluded that there was some justification for sub-species recognition of *conicopora* under *H. rubra*. Allozyme data suggested conspecifity but shell and geographic distributions indicated distinct taxa. Fifteen of 22 DNA microsatellite primers developed for use in *H. rubra* amplified a similar product in *H. conicopora* (Evans et al. 2001). This compares to the conservation of only 12 of the 22 markers in other temperate Australian species (*H laevigata, H.scalaris* and *H. roei*). Our short DNA sequences lend some support to the possibility of sub-species status for *conicopora*, however further research is required to resolve the issue.

The altered mobility of the *H. midae* mtCOI fragment run on polyacrylamide gels is most likely due to a conformation change. Conformational mutations attributed to sequence-specific variations are restricted to polyacrylamide gels and not seen on agarose gels (Singh et al. 1987). The location and conservation of this conformation variant requires further investigation. Its presence, however, raises a note of caution when using RFLPs as mobility variation of fragments seen on polyacrylamide gels may be misleading as they can be length or conformation polymorphisms. It is therefore recommended that species differentiation using the RFLP tests described here be run only on agarose gels.

The test described here fulfills the aim of our study to provide a relatively straightforward and cost-effective means for identifying several abalone species of commercial importance to Australia. Costs for any DNA-based analyses are not insignificant, but the PCR-RFLP technique is generally considered more cost-effective for routine species identification than alternatives such as direct DNA sequencing of the PCR product (e.g. Asensio et al. 2000). The opportunity to sequence a PCR product is of course still available for differentiation of individuals if problems arise following PCR-RFLP analysis.

To increase the potential value of this study to the sustainability and protection of abalone fisheries worldwide, additional species, particularly from Northern Hemisphere waters, need to be incorporated either into this test or a modified one, so that a single test is available for discrimination of all abalone species.

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TABLE 1. Expected restriction fragment lengths for eleven *Haliotis* species for the 193bp mtCOI fragment when cut with restriction enzymes *DdeI, HhaI, HinFI* and *HpaII*. The number in parenthesis represents the total number of individuals examined for each species that displays the given restriction pattern. Unique restriction profiles are shown in bold. *H. laevigata* numbers include the misidentified *H. scalaris* individual.

	CO1 Restriction Digestion Patterns			
	DdeI	*HhaI*	*HinFI*	*HpaII*
H. assinina	**7,50,137** (29) ***7,187*** (1)	193 (30)	34,159 (30)	3,42,72,76 (30)
H. australis	193 (10)	**79,114** (10)	193 (10)	**3,93,97** (10)
H. conicopora	193 (11)	42,151 (11)	34,159 (11)	3,42,51,97 (11)
H. iris	193 (10)	42,151 (10)	**19,174** (10)	3,190 (10)
H. laevigata	193 (63)	193 (62) 94,99 (1)	34,159 (62) 193 (1)	3,42,148 (63)
H. midae	193 (10)	42,151 (10)	34,159 (10)	3,42,148 (10)
H. roei	29,164 (10)	193 (10)	**6,34,97** (10)	45,51,97 (10)
H. rubra	193 (49) 29,164 (1)	42,151 (50)	34,159 (49) 193 (1)	3,42,51,97 (50)
H. scalaris	29,164 (19)	42,151 (19)	34,159 (19)	3,42,51,97 (19)
H. spadicea	193 (10)	42,151 (10)	**15,19,159** (10)	3,190 (10)
H. virginea	193 (10)	**42,57,94** (9) 42,151 (1)	193 (10)	3,190 (10)

TABLE 2. Expected restriction fragment lengths for ten *Haliotis* species for the 159 bp mtCOII fragment when cut with restriction enzymes *DdeI, EcoRV, HhaI, HpaII* and *RsaI*. *H. iris* did not amplify with these primers. The number in parenthesis represents the total number of individuals examined for each species that displays the given restriction pattern. Unique restriction profiles are shown in bold. *H. laevigata* numbers include the misidentified *H. scalaris* individual.

	CO2 Restriction Digestion Patterns				
	DdeI	*EcoRV*	*HhaI*	*HpaII*	*RsaI*
H. assinina	13,38,108 (30)	71,88 (30)	159 (30)	44,115 (30)	159 (30)
H. australis	13,15,131 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)
H. conicopora	13,15,131 (11)	159 (11)	58,101 (11)	159 (11)	159 (11)
H. iris	-	-	-	-	-
H. laevigata	**13,15,60,71** (63)	71,88 (63)	58,101 (63)	159 (63)	30,129 (63)
H. midae	13,15,131 (10)	159 (10)	159 (10)	159 (10)	159 (10)
H. roei	**13,146** (10)	159 (10)	159 (10)	44,115 (10)	30,129 (10)
H. rubra	13,15,131 (50)	159 (50)	58,101 (48) 159 (2)	159 (48) 44,115 (2)	159 (50)
H. scalaris	**13,71,75** (19)	159 (19)	159 (19)	159 (19)	30,129 (19)
H. spadicea	13,15,131 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)
H. virginea	13,38,108 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)

Figure 1. Electrophoretic analysis of undiluted (lanes 1 to 6) and diluted (x 10, lanes 9 to 14)) 193 bp mtCOI (upper image) and 159 bp mtCOII (lower image) PCR fragments for *H. rubra* tissue and mucous samples. M = 100 bp ladder. Samples in lanes are as follows: 1, 2, 9 & 10 muscle tissue; 3, 4, 11 & 12 fluid sample from plastic bag; 5, 6,13 & 14 mucous swab from plastic bag; 7 & 15 positive *H. rubra* DNA (x 20 dilution) control; 8 & 16 negative H_2O control.

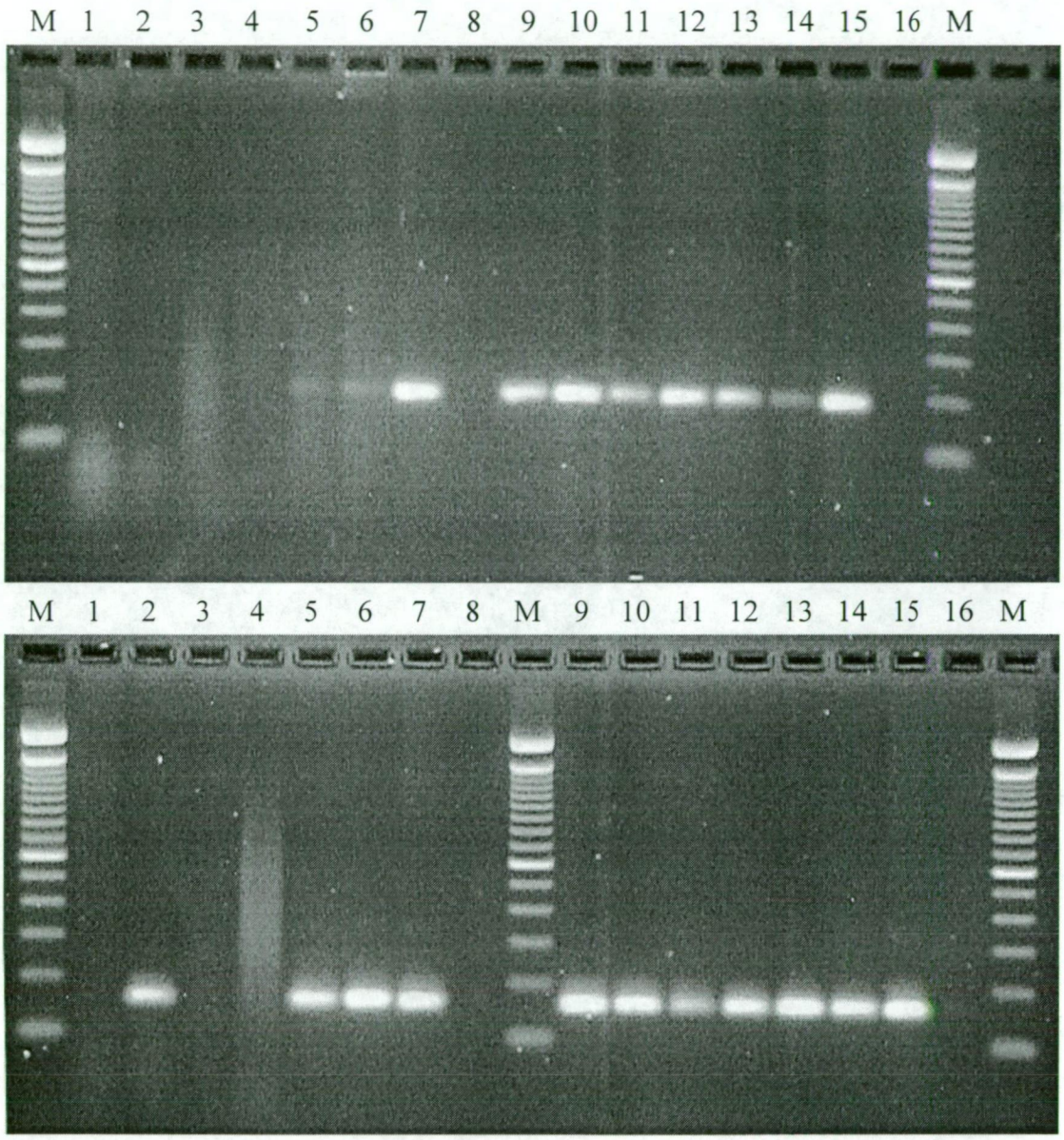


Figure 2 Sequence alignment of the 193 bp mtCOI fragment for ten abalone species. Primer sequences and cut sites for the four restriction enzymes *DdeI, HhaI, HinFI* and *HpaII* are included. (N = sequence data unclear whether C or T).

```
                1                                                  50                                                 100
H.rubra         CTGACATGGC TTTTCCTCGA CTAAATAATA TAAGATTCTG ACTACTCCCA CCCTCACTAA CCCTTCTATT AACATCGGGT GCTGTAGAAA GTGGTGCCGG
                                                  HinFI                                                              HpaII
H.conicopora    .......... .......... .......... ..........  .......... .......... .......... .......... .......... ..........
                                                  HinFI                                                              HpaII
H.laevigata     .C........ ...C...... .......... ..........  G..T...... ..T....... .T..GT.... G.....A... ..C....... .......T..
                                                  HinFI
H.scalaris      .C........ C..C...... ........C. .G........  ...T...... ..T....... .T..G..... ......A... .................. ..........
                                                  HinFI                                                              HpaII
H.assinina      .......A.. C..C..A... .....C..C. .G........  .........C ..A..C..C. .T..A..TC. .....C..G .....C..G. .......A..
                                                  HinFI                                  HpaII
H.roei          .......... C..C...... ..T..C.... ..........  ....C..... ..T....... .T..GT.... ......A... .......... ..........
                                                  HinFI HinFI                                                        HpaII
H.midae         .A.....A.. A..C...... ..T.....C. ..........  ...T..T..C ..A..TT.G. ....A..T.. ......A..G .......... .......A..
                                                  HinFI
H.spadicea      .C........ ...C.....  .C.....C. .G........  ...T...... ..T...T... .TT.A..C.. .......... .......... .......A..
                                  HinFI           HinFI
H.australis     .A.....A.. C.....C... ........C. .......T..  G..T..T..C ..A..T..C. .......TC. G.......C  .......... .C.......
                                                                                                    HhaI               HpaII
H.iris          .A..T..... .........  .C..C..C. .......T..  ......T..C ..A..C.... .T.....CC. ......A..A ..C....... .C.....A..
                                  HinFI
H.virginea      .G........ G..N..G... ..T..C.... .......T..  ...T.....T ..G..CT... .T..A..TC. C.....A..G ..C....... ...C..A..
                                                                                                                       HhaI
HALCO1-NG1      CIGACATRGC ITTYCCICGA CT--------- ----------  ---------- ----------  ---------- ---------- ---------- ----------
HALCO1-NG2      ---------- ---------- ---------- ----------  ---------- ----------  ---------- ---------- ---------- ----------
```

Figure 2 Continued..

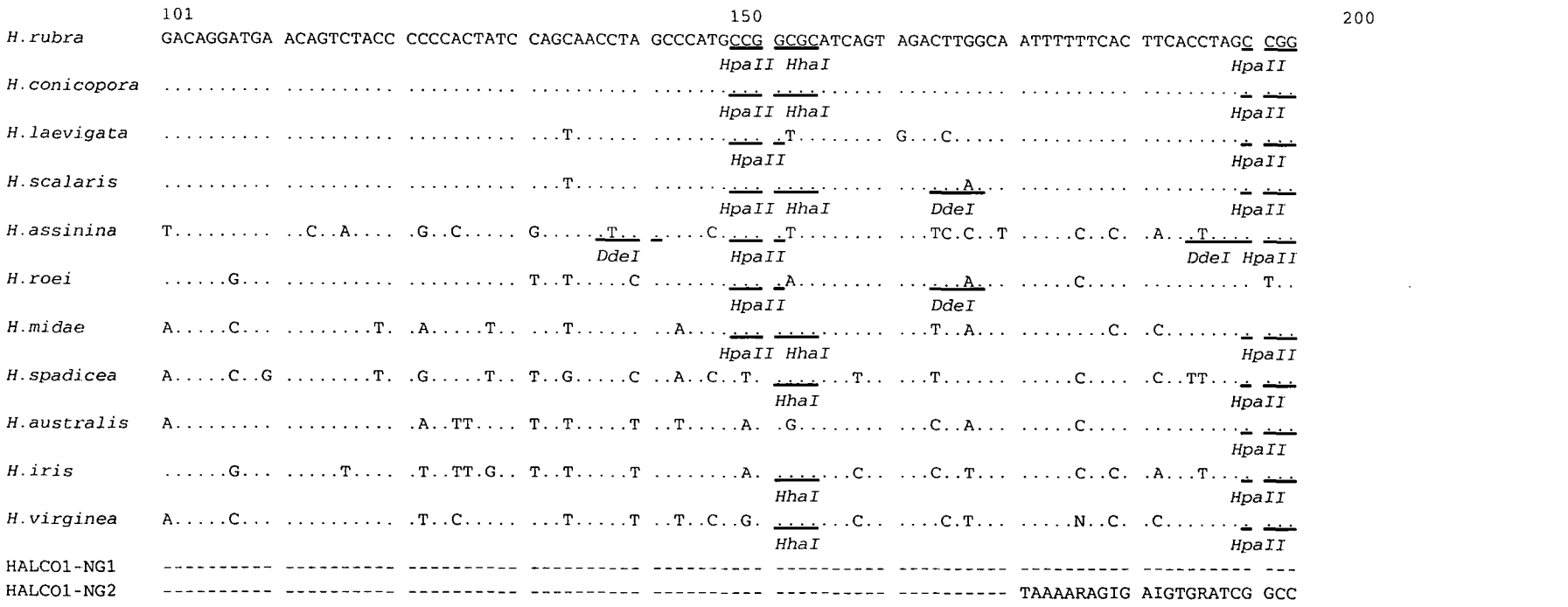


Figure 3. RFLP patterns on a non-denaturing polyacrylamide gel of the 193 bp mtCOI fragment for three abalone species produced with four restriction enzymes. Species 1 – *H. midae*, species 2 – *H. rubra*, species 3 – *H. laevigata*, M – 100 bp DNA ladder. Reduced mobilty in *H. midae* fragments suspected to be due to a conformational mutation.

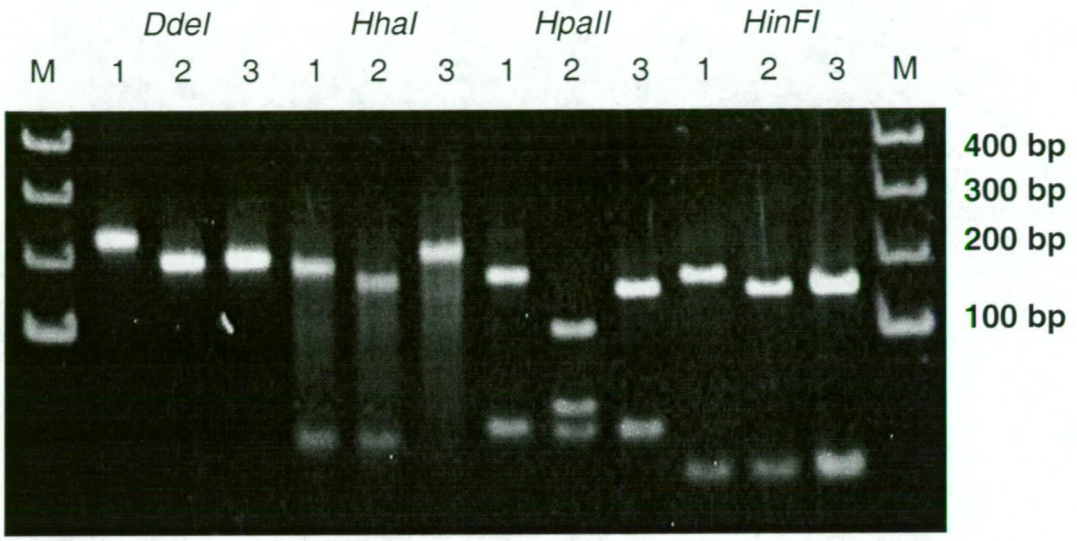
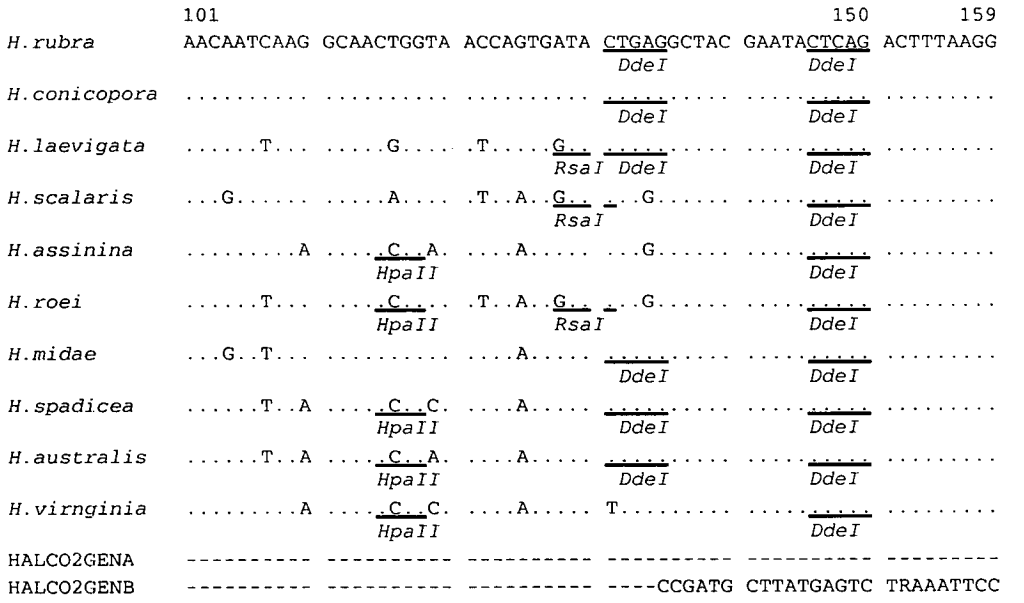


Figure 4. Sequence alignment of the 159 bp mtCOII fragment for ten abalone species. This fragment did not amplify for *H. iris*. Primer sequences and cut sites for the five restriction enzymes *DdeI, EcoRV, HhaI, HpaII* and *RsaI* are included. (1 = sequence data unclear whether C or T ; 2 = sequence data unclear whether C or A).

```
                1                                                  50                                                 100
H.rubra         CAATTTGAAC CATTCTACCA GCCATTATCC TTATTTTCCT CGCCCTACCA TCCTTGCGCC TCCTTTACCT ACTAGACGAA GTCGGTATAT CGTGCCTTCT
                                                                                  HhaI
H.conicopora    .......... .......... .......... .......... .......... ....C.... .......... .......... .......... ..........
                                                                                  HhaI
H.laevigata     .......... ......C... .......... .C..C..... .......... ....C.... .......... CT...T.... ..A..G.... .C........
                                                                                  HhaI                    DdeI          EcoRV
H.scalaris      .......... C.....C... .......... .C..C..... A.....G... .....A.... .......... CT...T.... ..A..A.... .C........
                                                                                                          DdeI
H.assinina      .......... T......... .....C.... .G..C..T. A.....T... ..TC.C..A. .T..A..... .......... ..T..G.... .T.....CT.
                                                    DdeI                                                                EcoRV
H.roei          .......... T.....C... .......... .C..C..... A.....C... .....A.... .T........ .......... ..A........ .C.....C..
H.midae         .......... C.....C... ..T........ .A..C..... ......C... ..TC.A..A. ....C..... C......... ..G........ .A..T..CT.
H.spadicea      .......... 1.....C... ..T........ ..G.C..... ......T... .....A..A. .......T.. ......T... ..A.....G. .A..T...T.
H.australis     .......... 1.....2... .....C..TT .A........ A.....G..G ...C.A..A. .......... C.....T... .......... .A.....CT.
H.virnginia     .......... ..C....... .....C.... .A..C..T. A.....C..C ..AC.T..A. .T........ T.....T..G .....A.... .A.....A..
                                                    DdeI
HALCO2GENA      CAATYTGAAC YATTCTMCCA GC-------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
HALCO2GENB      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

Figure 4. Continued..



Chapter 9 General Discussion

Various genetic markers are available for fisheries and aquaculture research. Each marker type provides a different approach to answer the range of questions posed by managers of wild fisheries, and hatchery and grow-out managers of aquaculture. This thesis is the first comprehensive evaluation of the utility of a relatively new class of molecular marker, the microsatellite, for abalone research.

The development of a suite of 22 microsatellite DNA markers in the Australian blacklip abalone, *Haliotis rubra* (Chapter 2) has provided new tools for the investigation of genetic variation in both hatchery and wild stocks of abalone. I have demonstrated the application of such markers for examinations of wild and cultured abalone populations within south-eastern Australia (Chapters 4 and 7) and that they may also be optimised for the analysis of genetic variation in other abalone species world-wide (Chapter 3, 6 and 7). The markers I developed have also been used in other studies on Australian *H. rubra* and *H. laevigata* species (Conod *et al.* In Press; Personal Communication: Nick Elliott, CSIRO Marine Research), and are being used in a large collaborative population structure study of *H. rubra* (Personal Communication: Nick Elliott, CSIRO Marine Research).

Studies of microsatellite markers in other taxa have shown that their conservation in related species and genera is variable. Research in marine mammals has shown that the sequence flanking microsatellite repeats in cetaceans is unusually well conserved (Schlötterer *et al.* 1991). Microsatellite conservation in the Meliaceae family (hardwood) however, ranges from species-specific markers, to family-wide markers (White and Powell 1997). My investigation of microsatellite marker conservation within the genus *Haliotis* (Chapter 3) has revealed that the number of markers shared between two species of abalone is related to the phylogenetic relationship of those two species. Closely related abalone species are more likely to share a microsatellite locus, than distantly related species (Chapter 3). Technical factors must also be considered when attempting to optimise microsatellite loci in species other than the one in which they were designed. Large scale studies of genetic variation require not only the presence of amplifying loci, but the reliable amplification of moderately polymorphic loci, that can be easily and reliably scored using the system available in each laboratory.

I have shown that it is possible for *H. rubra* markers to be used in a range of other abalone species with very little optimisation time. The time and resources required to develop new markers for each species is great, yet with little optimisation time, I was able to transfer three *H. rubra* microsatellites to an examination of genetic variation in hatchery and wild stocks of the South African perlemoen, *H. midae* (Chapter 6 and 7). This research would not have been

possible had the added costs of marker development in that species been necessary. Similar research into the genetic variation of greenlip abalone, *H. laevigata* around Australia was also made possible by the optimisation of *H. rubra* microsatellites (Personal Communication: Nick Elliott, CSIRO Marine Research, Hobart).

There are presently 86 reported microsatellite loci available for abalone species worldwide (Table 9-1). Microsatellite DNA markers have been developed for abalone species in the Unites States of America (Kirby *et al.* 1998), Canada (Miller *et al.* 2000), Japan (Sekino *et al.* 1999) and Australia (Huang and Hanna 1998; Selvemani *et al.* 2000; Evans *et al.* 2000; Evans *et al.* in press). With microsatellite markers already developed in six abalone species, from four distant countries, it is likely that genetic research in related species may be possible, at least on a pilot scale, without the need for further marker development (Chapter 3). The development of new markers however, is required to provide sufficient loci for genetic mapping projects (eg. As 2n = 36 in many abalone species, over 150 markers would be required to achieve an average of 5 markers located on each chromosome), and more reliability of amplification, polymorphism and gel scoring for genetic variation and pedigree studies.

Table 9-1 Microsatellite loci available for abalone species worldwide.

Species	loci	Author
Haliotis rufescens	1	Kirby *et al.* 1998
Haliotis rubra	3	Huang and Hanna 1998
Haliotis discus discus	37	Sekino *et al.* 1999
Haliotis rubra	22	Evans *et al.* 2000; Evans *et al.* in press
Haliotis asinina	11	Selvamani *et al.* 2000
Haliotis kamtschatkana	12	Miller *et al.* 2000

In order to resolve questions of abalone population structure, such as those posed in Chapters 4 and 6, DNA markers of suitably high variability are required. Microsatellite DNA markers are highly variable, co-dominant markers, that are common throughout the genome of most eukaryotes (Lanzaro *et al.* 1995). These properties have led to them being described as an ideal marker for population genetic research (Bowcock *et al.* 1994). Whilst genetic variation of abalone populations has previously been examined at allozyme loci (eg. Brown 1991; Sweijd 1999; Hancock 2000) and in mitochondrial DNA (eg. Jiang *et al.* 1995; Sweijd 1999; Conod *et al.* In Press), there have been only two other reported examinations of abalone population genetics, both in *H. rubra* that have utilised variation at microsatellite loci (Huang *et al.* 2000; Conod *et al.* In Press). In Chapter 4, I describe the most comprehensive

microsatellite study in abalone reported to date, it reveals differentiation of Tasmanian and mainland Australian *H. rubra* populations, but no heterogeneity within each group. I also present data that contradicts the conclusions of high levels of inbreeding and population differentiation reported for this species by Huang *et al.* (2000). My data suggests that the high levels of homozygosity reported by Huang *et al.* (2000) are more likely to be due to technical problems in data collection than the high levels of inbreeding that they suggest. My examination of genetic variation at three microsatellite loci in *H. midae* populations in South Africa (Chapter 6) supports previous evidence from mitchondrial DNA haplotypes (Sweijd 1999) of major differentiation between east and west coast stocks.

The highly polymorphic nature of microsatellites makes them a powerful tool for the evaluation of hatchery effects on genetic variation. A loss of rare alleles was recorded in farmed abalone samples of *H. rubra* and *H.midae* examined in Tasmania and South Africa respectively, although no decrease in heterozygosity was observed in either species. Change in the identity and frequency of the most common allele was also observed in Tasmanian farmed abalone samples. Such changes in microsatellite and allozyme allelic diversity have been reported in other species (eg. Atlantic Salmon - Norris *et al.* 1999; Brown trout - Vuorinen 1984), and although changes at allozyme loci have been reported in *Haliotis iris* in New Zealand (Smith and Conroy 1992), my research provides the first examination of hatchery effects on genetic variation of farmed abalone at microsatellite loci (Chapter 7).

The loss of genetic variation within farmed abalone in the form of rare allele loss, and dramatically altered allele frequencies has implications to the fitness of the farm stocks. For example, genetic variation in the Atlantic silverside, a marine fish, has been linked to traits such as food consumption rate and growth efficiency (Present and Conover 1992), winter survival (Schultz *et al.* 1998), and even spawning cues (Conover, unpublished data cited in Conover 1998). Losses, such as the ones outlined in Chapter 7, could be amplified in future generations by uncontrolled breeding from hatchery reared broodstock. If the abalone culture industry is going to move towards a closed breeding cycle, as it appears to be doing, then it will be necessary to embrace available molecular technologies to ensure that declines in genetic variation are not detrimental to the performance of farm stocks. The development of an industry-wide genetic breeding program is an option currently being considered for the Australian abalone culture industry. The implementation of programs such as these is an important process in the coming of age of the industry, as the long term gains offered by genetic improvement programs appear to far outweigh the short term costs.

An excess of observed homozygotes, as I report in Chapters 4 and 6 can be due to high levels of inbreeding or sex-linkage of the markers (Huang *et al.* 2000), to the presence of null alleles, or the incorrect scoring of alleles. In Chapter 5, I confirm the presence of null alleles at two *H.*

rubra microsatellite loci as the most likely cause of the observed disequilibria, and examine reasons for the non amplification of alleles at these loci. Evidence that "null homozygotes" are not always due to the presence of a mutation(s) within the primer binding site as previously suggested (eg. McGoldrick *et al.* 2000) is also presented. The presence of null alleles in population genetic research is not considered to be a major problem. If null alleles are distributed evenly through all samples examined then their presence will not alter or mask any differentiation that may be present within the species, this means that null alleles can be effectively ignored for population analysis purposes. The presence of null alleles at loci used in structured breeding programs however, can result in difficulties in the assignment of parentage (Ede and Crawford 1995; Eggleston-Stott *et al.* 1997). For this reason, markers developed for future breeding programs should be tested on known family crosses to limit the possibility of using markers containing null alleles, and to understand the inheritance of null alleles between generations. Assigning parentage to the progeny of batch spawnings will prove to be problematic if these limitations are not considered.

Microsatellite markers developed for *H. rubra* may not be useful for studies of genetic variation in *H. rubra*, *H. laevigata* hybrid abalone (Chapter 7). An examination of the hybrid progeny of three supposed single pair crosses revealed an excess of alleles, in addition to those expected to be contributed by the two parents, in two of the three families lines. Whilst it may be possible to account for the presence of extra male alleles in the progeny through sperm contamination at fertilisation, the accidental mixing of eggs from more than the intended female parent is unlikely. Furthermore, the inheritance of one allele differed dramatically from Mendelian expectations in the hybrid progeny of the one successful single parent cross. The proliferation of anomalies in the genetic data obtained for *H. rubra*, *H. laevigata* hybrid abalone therefore indicate that more extensive research is required into the affect of cross-species hybridisation on the mutation rates and inheritance of highly polymorphic microsatellite loci.

Highly polymorphic markers are not always the best choice for genetic research. Where differentiation at the species level is required, a less variable marker is able to provide a simpler method of differentiation. In chapter 8, I discuss the development of an identification technique for 11 Southern Hemisphere species of abalone. This technique utilises variation within two regions of mitochondrial DNA, and has been extensively tested by fellow researchers at CSIRO Marine Research, Hobart, and is now being tested by the Tasmania Police forensic science group. An extension of the technique to include all abalone species is envisaged, and required to help reduce the impact of international abalone poaching. The problem of abalone poaching is a global one, and my research was developed with strong collaboration between South African and Australian scientists and enforcement agencies. Similar collaborations should be encouraged for the benefit of all abalone resources.

The genetic data collected to date indicate that the Tasmanian blacklip abalone population is effectively a single panmictic stock that is partially isolated from stocks around southern mainland Australia. In order to determine the structure of stocks along the southern coastline of mainland Australia, the microsatellite study in Chapter 4 of this thesis should be expanded to examine samples from the complete range of the species. This range should spread as far as south-west Western Australia as *H. conicopora* is considered to be a sub-species of *H. rubra*. Further consideration should also be given to this question of species identity given the similarity of sequence data obtained for these two species.

The primary growth area of genetic research as I see it, in the next five years, will be in the field of abalone aquaculture. The markers I have developed provide a good starting point for the establishment of structured, marker assisted genetic breeding programs in *H. rubra* and *H. laevigata*. The continued expansion and development of abalone aquaculture however, relies on additional marker development for genetic mapping and reliable pedigree analysis, and an industry wide acceptance of the benefits that controlled genetic breeding programs will provide.

In conclusion, new microsatellite markers have been developed and successfully applied to a series of genetic questions in both hatchery and natural populations of abalone, with particular emphasis on *Haliotis rubra* and *H. midae*. This research provides an insight into the many ways that genetic markers can be used to assist in the management of wild abalone fisheries, and to increase the yield and efficiency of abalone farms in the future. In particular I have:

1. Created a partial genomic library of *H. rubra* DNA, and developed 22 primer pairs for the amplification of 21 microsatellite loci.
2. Examined the conservation of those markers in 12 other species of abalone, and shown a relationship between marker conservation and reported phylogenetic relationships.
3. Found little evidence for differentiation within Tasmanian *H. rubra* stocks, but shown them to be separate from stocks around south-eastern mainland Australia.
4. Confirmed the separation of South African *H. midae* stocks on either side of Cape Agulhas.
5. Confirmed the presence of null alleles at two *H. rubra* microsatellite loci as a cause of Hardy-Weinberg disequilibrium, but shown that this is not simply due to primer site mutation.
6. Demonstrated a dramatic loss of rare alleles and changes in allele frequency in first generation abalone hatchery stocks.
7. Provided a species identification protocol for the separation of 11 Southern Hemisphere species.

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Appendix A Allele frequencies at eight microsatellite loci in samples of *H. rubra* from south-eastern Australia. Number of individuals in parentheses.

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***CmrHr* 1.14**					
251	0.045	0.034	0.069	0.060	0.066	0.044	0.060	0.113	0.083	0.089
253	0.006	-	-	-	-	-	-	-	-	-
259	0.826	0.879	0.875	0.760	0.779	0.874	0.832	0.766	0.800	0.768
261	0.112	0.080	0.050	0.095	0.139	0.071	0.076	0.073	0.089	0.105
263	0.006	-	-	0.020	-	-	0.011	-	0.006	-
265	-	-	-	-	-	-	0.005	0.008	-	-
267	0.006	-	0.006	0.030	0.008	0.011	0.011	0.016	0.011	0.011
269	-	-	-	0.010	-	-	-	-	0.006	0.016
271	-	0.006	-	0.005	-	-	-	0.024	-	-
275	-	-	-	0.010	-	-	-	-	-	-
277	-	-	-	-	0.008	-	-	-	0.006	-
283	-	-	-	-	-	-	0.005	-	-	-
289	-	-	-	0.010	-	-	-	-	-	0.005
293	-	-	-	-	-	-	-	-	-	0.005
					***CmrHr* 1.24**					
212	-	-	-	-	-	0.006	-	-	-	-
216	0.027	-	0.006	0.005	0.008	0.011	0.027	-	0.011	0.016
220	-	-	-	0.005	-	-	-	0.008	-	-
222	0.846	0.855	0.904	0.815	0.762	0.800	0.824	0.894	0.826	0.797
224	0.088	0.075	0.045	0.090	0.131	0.106	0.115	0.061	0.103	0.146
226	0.027	0.065	0.034	0.060	0.074	0.044	0.011	0.030	0.043	0.036
228	0.011	0.005	0.006	0.025	0.025	0.028	0.022	0.008	0.005	0.005
236	-	-	0.006	-	-	0.006	-	-	0.011	-
					***CmrHr* 1.25**					
290	0.037	-	0.009	0.006	-	-	0.030	-	0.008	-
292	-	-	-	-	-	-	-	0.024	-	-
294	-	0.013	0.009	0.022	0.010	0.013	0.023	0.012	-	0.018
296	0.007	-	-	-	0.030	0.013	0.008	0.012	-	0.018
298	0.007	0.033	0.036	-	-	-	-	-	-	-
300	0.119	0.079	0.064	0.112	0.110	0.090	0.045	0.048	0.070	0.018
302	0.090	0.053	0.209	0.180	0.230	0.288	0.182	0.202	0.227	0.220
304	0.104	0.105	0.100	0.112	0.020	0.160	0.106	0.107	0.094	0.134
306	0.075	0.026	0.109	0.135	0.120	0.038	0.174	0.131	0.141	0.110
308	0.060	0.072	0.027	0.051	0.120	0.147	0.076	0.155	0.070	0.067
310	0.022	0.033	0.045	0.045	0.020	0.032	0.068	0.024	0.070	0.030
312	0.045	0.033	0.027	0.073	0.090	-	0.023	0.048	0.016	0.049
314	0.075	0.046	0.064	0.011	0.030	0.013	0.061	0.048	0.039	0.006
316	0.015	0.026	0.018	0.011	-	0.051	0.023	0.024	0.023	0.024
318	0.030	0.033	-	-	0.030	0.013	-	-	0.008	0.012
320	0.045	0.039	0.045	0.006	0.030	0.006	0.023	0.012	0.023	0.030
322	-	0.026	0.027	0.039	0.020	-	-	0.012	0.008	0.006

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***CmrHr* 1.25**					
324	0.037	0.026	0.045	0.011	0.020	0.013	0.008	0.012	0.008	0.006
326	0.007	0.046	-	0.017	0.020	-	0.008	-	0.039	0.024
328	0.015	0.026	-	-	-	0.026	0.023	0.024	-	0.049
330	-	0.046	0.027	-	-	-	0.015	-	0.008	0.006
334	0.037	0.033	0.018	0.028	-	-	0.008	0.024	0.023	0.043
336	0.030	0.007	0.009	0.006	-	-	0.015	-	0.016	0.006
338	0.037	0.026	-	0.006	0.020	0.013	0.008	-	0.023	-
340	0.037	0.007	-	0.006	0.030	0.019	-	0.024	0.031	0.018
342	-	0.020	-	-	-	0.006	0.015	-	0.016	0.018
344	0.015	0.026	0.036	0.028	0.010	0.006	-	-	-	0.006
346	0.015	0.026	0.009	0.006	0.010	0.006	0.008	0.024	0.016	-
348	0.015	0.020	-	-	-	-	-	-	-	-
350	-	0.007	0.018	0.006	0.010	0.013	0.008	-	-	-
352	-	0.020	0.009	0.017	-	0.013	0.008	-	-	0.006
354	0.015	0.013	-	-	-	-	0.008	-	-	0.012
356	0.007	0.013	-	0.011	-	-	0.023	0.012	-	-
358	-	0.013	0.018	0.022	-	-	-	-	0.008	-
360	-	-	0.018	-	0.020	-	-	0.024	-	0.030
362	-	0.007	-	-	-	0.019	-	-	0.008	-
364	-	-	-	0.006	-	-	-	-	-	0.006
366	-	-	-	0.011	-	-	0.008	-	0.008	0.024
368	-	-	-	0.017	-	-	-	-	-	-
					***CmrHr* 2.9**					
158	0.108	0.078	0.091	0.155	0.169	0.213	0.120	0.147	0.205	0.141
166	0.205	0.224	0.250	0.144	0.144	0.184	0.098	0.155	0.120	0.114
168	0.017	0.010	0.011	0.026	-	0.023	0.033	0.034	0.024	0.022
172	-	-	0.011	-	-	-	-	-	-	-
176	0.017	0.010	0.006	-	-	-	0.033	-	-	-
178	0.006	0.010	0.006	0.010	0.025	0.029	-	0.009	0.006	-
180	0.023	0.016	0.011	0.021	0.025	0.011	0.016	-	0.012	0.005
182	0.017	0.026	0.028	0.005	0.008	-	0.027	0.017	0.012	0.005
184	0.023	0.057	0.028	0.052	0.034	0.023	0.027	0.026	0.042	0.043
186	0.028	0.063	0.017	0.057	0.068	0.052	0.060	0.052	0.054	0.082
188	0.045	0.057	0.017	-	-	0.011	0.016	0.017	-	0.022
190	0.080	0.057	0.040	0.031	0.068	0.023	0.011	0.017	0.024	0.016
192	0.034	0.031	0.034	0.062	0.042	0.029	0.027	0.052	0.078	0.071
194	0.045	0.036	0.051	0.010	0.025	0.023	0.065	0.017	0.018	0.011
196	0.034	0.016	0.011	0.010	0.017	0.023	0.005	0.052	0.024	0.005
198	0.017	0.005	0.023	0.046	0.042	0.029	0.060	0.034	0.066	0.027
200	0.028	-	0.057	0.021	-	0.040	0.033	0.017	0.018	0.022
202	0.023	0.016	-	0.062	0.034	0.017	0.027	0.060	0.012	0.054
204	0.017	0.047	0.017	0.021	-	0.023	0.005	0.009	0.030	0.016
206	0.017	0.031	0.017	0.021	0.076	0.023	0.038	0.034	0.018	0.016
208	0.017	0.036	0.040	0.021	0.034	0.011	0.011	0.017	0.036	0.027

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***CmrHr*2.9 cont..**					
210	0.023	0.026	0.017	0.015	-	0.017	0.016	0.009	0.012	0.027
212	0.023	0.016	0.023	0.021	0.008	0.011	0.049	0.043	0.018	0.027
214	0.017	0.005	0.011	0.021	0.025	0.023	0.038	0.017	0.048	0.038
216	0.017	0.021	0.006	0.026	0.025	0.006	0.022	0.026	0.024	0.027
220	0.017	0.031	0.040	0.046	0.025	0.011	0.038	0.009	0.012	0.016
222	0.006	0.010	0.023	-	0.034	0.023	0.005	0.009	0.024	0.016
224	0.006	0.010	0.023	0.010	-	0.023	0.027	0.026	0.006	0.016
226	0.017	0.021	0.023	0.015	0.025	0.023	0.022	0.017	0.018	0.027
228	0.017	0.005	0.028	0.021	-	0.011	0.027	0.017	-	0.033
230	0.006	-	0.006	0.015	0.017	0.011	0.005	0.026	0.012	0.011
232	0.006	0.016	0.011	0.010	-	-	0.005	0.009	0.006	0.005
234	0.017	0.005	-	-	0.017	0.011	0.011	-	-	0.016
236	0.011	-	0.006	0.005	-	-	0.005	0.009	0.006	0.005
238	0.006	-	0.006	0.005	-	-	-	-	0.006	0.016
240	0.006	0.005	0.011	-	-	0.006	-	0.017	-	-
242	-	-	-	-	-	0.006	0.005	-	-	-
244	-	-	-	0.005	-	0.006	-	-	-	0.011
246	-	-	-	0.005	-	0.006	0.011	-	0.006	-
248	-	-	-	0.005	0.008	0.017	-	-	-	0.005
262	0.006	-	-	-	-	-	-	-	-	-
					***CmrHr*2.14**					
200	-	0.005	-	-	-	-	-	0.016	-	-
208	0.011	0.005	0.012	0.016	-	0.007	-	0.016	0.006	0.011
212	0.028	0.026	0.024	0.032	0.025	0.049	0.056	0.016	0.056	0.011
216	0.133	0.107	0.118	0.112	0.107	0.090	0.099	0.081	0.112	0.087
220	0.011	0.005	0.012	-	0.016	-	0.037	0.016	0.006	0.005
224	0.444	0.561	0.506	0.537	0.459	0.521	0.420	0.419	0.438	0.489
228	0.161	0.117	0.194	0.165	0.205	0.153	0.247	0.274	0.206	0.179
232	0.011	0.020	0.012	0.011	0.008	0.007	0.019	0.016	0.025	0.043
236	0.194	0.148	0.118	0.128	0.180	0.174	0.123	0.145	0.138	0.174
244	0.006	0.005	0.006	-	-	-	-	-	-	-
252	-	-	-	-	-	-	-	-	0.013	-
					***CmrHr*2.26**					
168	0.041	0.071	0.038	0.126	0.172	0.117	0.104	0.103	0.055	0.089
180	0.012	0.005	0.006	-	-	-	-	-	-	-
184	0.012	0.010	0.025	0.011	0.016	0.011	0.007	-	0.043	0.005
188	0.140	0.117	0.139	0.105	0.172	0.106	0.160	0.151	0.128	0.153
192	0.209	0.276	0.278	0.216	0.230	0.283	0.278	0.262	0.159	0.153
196	0.180	0.138	0.152	0.126	0.098	0.083	0.097	0.095	0.177	0.100
200	0.140	0.117	0.139	0.116	0.107	0.078	0.111	0.079	0.171	0.132
204	0.169	0.163	0.089	0.100	0.090	0.111	0.111	0.183	0.128	0.200
208	0.041	0.071	0.038	0.105	0.049	0.089	0.049	0.079	0.091	0.047
212	0.041	0.005	0.025	0.021	0.025	0.039	0.049	0.024	0.018	0.074

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***CmrHr* 2.26**					
216	0.006	0.015	0.019	0.037	0.016	0.011	0.014	0.016	0.012	0.032
220	0.012	0.010	0.013	-	0.016	0.028	-	-	-	-
224	-	-	0.025	0.021	-	-	0.007	-	0.018	0.005
228	-	-	-	-	0.008	0.017	-	0.008	-	0.011
232	-	-	0.013	0.011	-	0.028	0.014	-	-	-
240	-	-	-	0.005	-	-	-	-	-	-
					***CmrHr* 2.30**					
261	-	-	-	-	-	0.017	-	-	-	0.005
263	-	-	-	-	-	0.006	-	-	-	-
265	-	0.005	-	-	-	-	-	-	-	-
267	-	-	-	-	-	-	-	-	0.006	-
269	-	-	0.011	0.005	-	0.006	-	-	0.013	0.005
271	-	-	0.006	0.005	-	-	-	-	-	-
273	0.005	0.005	-	-	-	-	-	-	-	0.005
275	0.016	0.005	0.006	-	-	0.006	-	-	0.019	-
277	0.005	0.020	-	-	-	0.017	-	0.016	0.038	-
279	0.005	-	-	-	-	-	-	-	-	-
281	-	-	0.011	0.005	-	0.017	0.006	-	-	-
283	0.016	0.005	0.034	-	0.008	0.034	0.017	0.049	0.044	-
285	0.011	-	0.011	0.015	0.017	0.017	0.006	0.033	0.013	0.016
287	0.022	0.015	0.011	-	-	-	-	0.016	-	0.021
289	0.044	0.066	0.063	0.030	0.017	0.067	0.017	0.025	0.038	0.016
291	0.132	0.136	0.057	0.020	0.050	0.056	0.023	0.082	0.089	0.016
293	0.055	0.076	0.040	0.060	-	0.022	0.006	0.098	0.057	0.016
295	0.132	0.126	0.086	0.025	0.008	0.107	0.011	0.107	0.070	0.010
297	0.137	0.111	0.098	0.055	0.017	0.073	0.086	0.057	0.076	0.042
299	0.033	0.025	0.052	0.075	0.058	0.045	0.057	0.008	0.032	0.083
301	0.027	0.061	0.075	0.055	0.092	0.067	0.115	0.057	0.013	0.104
303	0.027	0.035	0.023	0.070	0.067	0.006	0.057	0.025	0.025	0.068
305	0.071	0.051	0.052	0.025	0.100	0.090	0.092	0.057	0.025	0.057
307	0.022	0.020	0.017	0.035	0.033	0.022	0.029	0.025	0.038	0.057
309	0.011	0.015	0.011	0.035	0.075	0.017	0.023	0.025	0.013	0.031
311	0.022	0.020	-	0.030	0.033	0.006	0.069	-	0.006	0.031
313	0.005	0.025	-	0.030	0.092	0.006	0.034	-	0.006	0.047
315	-	0.010	0.011	0.030	0.033	0.017	0.006	0.008	0.063	0.010
317	0.016	0.010	-	0.030	0.008	0.051	0.034	0.025	0.006	0.031
319	0.027	0.040	0.063	0.010	0.025	0.017	0.011	0.049	0.089	-
321	0.016	-	0.006	0.010	0.008	0.006	0.011	-	0.013	0.005
323	0.011	0.015	0.023	0.035	0.025	0.017	0.029	0.025	0.019	0.010
325	0.011	0.005	-	0.030	-	0.006	0.017	0.008	0.006	0.026
327	-	-	-	0.020	0.025	-	0.034	-	0.006	0.042
329	-	0.005	0.011	0.040	0.008	0.017	0.040	0.008	0.013	0.016
331	-	-	0.011	0.005	-	-	0.006	-	-	-
333	0.016	0.010	-	0.010	-	0.006	0.011	-	0.006	0.005

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***CmrHr* 2.30**					
335	-	0.005	0.011	0.005	-	0.011	0.006	0.008	-	0.005
337	-	0.005	-	0.010	-	-	0.029	-	0.038	0.010
341	0.005	-	0.017	0.010	0.017	0.006	0.011	-	0.006	0.016
343	0.016	0.010	0.006	0.010	0.017	0.006	0.006	0.008	0.013	0.021
345	0.005	0.015	0.006	0.005	0.025	0.011	-	0.008	0.006	0.010
347	-	-	0.006	0.010	-	0.011	-	-	0.013	0.005
349	-	-	-	0.005	0.008	-	0.006	-	-	0.005
351	0.011	-	0.023	0.005	0.017	0.006	0.006	0.016	0.006	0.016
353	0.011	0.005	0.023	0.010	-	0.006	0.006	0.025	0.019	-
355	-	0.010	0.017	0.005	0.008	-	0.006	-	0.013	0.010
357	-	0.020	0.029	0.005	0.008	0.017	-	0.016	0.013	0.005
359	0.011	-	-	0.010	-	0.017	0.006	-	-	0.005
361	0.005	-	0.011	0.005	0.017	-	0.011	0.016	-	0.016
363	-	-	0.006	0.010	-	-	0.023	0.008	-	0.016
365	0.011	0.005	-	0.015	0.025	-	-	-	0.006	0.021
367	-	-	0.006	0.010	0.008	0.022	-	0.008	-	0.010
369	0.005	0.005	0.011	0.005	-	0.028	-	-	-	0.005
371	-	-	-	0.015	-	-	0.017	-	0.006	-
373	-	-	0.011	0.020	0.008	-	-	0.025	-	0.010
375	0.005	-	-	0.005	-	0.011	0.006	0.008	0.006	0.010
377	0.011	-	0.017	-	0.025	-	0.006	-	0.006	0.010
379	-	-	-	0.025	-	0.011	0.006	0.008	-	0.005
381	-	-	-	-	-	-	-	0.016	-	-
385	-	-	0.006	0.005	0.017	-	-	0.008	0.006	0.010
389	-	-	-	-	-	-	-	0.016	-	-
					***rub*CA**					
110	-	0.005	-	0.015	0.016	0.012	0.033	0.008	-	0.036
114	0.006	-	-	-	-	0.006	-	-	-	-
118	0.267	0.137	0.171	0.305	0.246	0.285	0.255	0.164	0.293	0.286
122	0.011	0.011	0.012	0.025	0.008	0.006	0.022	-	0.017	0.005
126	0.006	0.011	0.012	0.020	0.025	0.006	0.016	0.008	-	0.031
128	0.011	0.011	0.030	0.005	0.016	-	-	0.016	0.011	0.021
130	-	-	0.006	-	-	0.006	-	-	-	-
132	-	-	-	-	0.008	-	0.005	-	-	0.005
134	0.017	0.022	-	0.015	0.008	0.012	0.011	0.016	0.023	0.021
136	-	-	-	0.005	0.008	-	0.005	-	0.011	-
138	-	0.011	0.012	0.015	0.008	0.017	0.005	0.025	0.011	0.005
140	0.006	0.038	0.030	-	0.008	-	0.016	0.008	0.017	-
142	0.033	0.022	0.012	0.045	0.008	0.029	0.011	0.049	0.034	0.021
144	0.017	0.038	0.030	0.065	0.066	0.041	0.022	0.082	0.034	0.052
146	-	0.038	0.018	0.010	-	0.017	0.011	0.008	0.006	0.010
148	0.039	0.033	0.079	0.020	0.025	0.023	0.054	0.025	0.040	0.036
150	0.011	0.016	0.006	0.015	0.008	0.017	0.005	0.016	0.017	0.026
152	0.067	0.077	0.067	0.020	0.049	0.047	0.082	0.074	0.052	0.042

Allele (bp)	*Kiama* (93)	*Jervis Bay* (100)	*Pearl point* (93)	*Georges Rocks* (100)	*Trump. Corner* (61)	*Curio Bay* (92)	*One Tree Point* (93)	*George III Reef* (68)	*Sterile Island* (93)	*Church Rocks* (96)
					***rub*CA cont..**					
154	0.050	0.033	0.012	0.005	0.033	0.017	0.011	0.025	0.011	0.005
156	0.061	0.016	0.067	0.030	0.049	0.029	0.038	0.049	0.046	0.026
158	0.094	0.115	0.104	0.105	0.090	0.093	0.098	0.057	0.080	0.052
160	0.022	0.049	0.073	0.035	0.074	0.041	0.033	0.090	0.040	0.052
162	0.033	0.038	0.024	0.015	0.041	0.047	0.033	0.057	0.029	0.052
164	0.078	0.044	0.073	0.030	0.041	0.070	0.098	0.041	0.046	0.063
166	0.017	0.011	0.018	0.025	0.016	0.029	0.022	0.049	0.017	0.021
168	0.044	0.038	0.024	0.035	0.008	0.052	0.027	0.033	0.052	0.031
170	0.017	0.027	0.012	0.020	0.041	0.017	0.011	0.008	0.011	0.016
172	0.033	0.038	0.006	0.005	0.008	0.012	0.016	0.025	0.006	0.010
174	0.011	0.027	-	0.025	0.025	0.006	0.016	0.016	0.023	0.026
176	0.017	0.011	0.024	0.020	0.016	0.012	0.011	0.008	0.023	0.005
178	-	0.022	0.018	0.005	-	0.006	0.016	0.008	0.006	0.021
180	-	0.016	0.024	0.010	0.016	0.006	-	-	0.011	-
182	0.011	0.016	0.012	-	-	-	0.005	-	0.006	-
184	-	0.011	-	-	0.008	-	-	-	0.006	0.010
186	0.011	-	0.006	0.005	-	-	-	-	-	-
188	-	-	-	0.005	-	0.006	-	-	0.006	-
190	0.006	0.005	-	0.015	-	0.006	-	-	-	0.005
192	-	-	-	0.015	0.008	-	0.005	0.016	0.011	0.005
194	-	0.005	-	0.005	0.008	0.017	-	0.016	-	-
196	-	-	0.006	0.010	0.008	-	-	-	-	-
198	0.006	-	0.006	-	-	0.006	-	-	-	-
202	-	-	-	-	-	0.006	-	-	-	-
208	-	-	-	-	-	-	0.005	-	-	-

Appendix B Allele frequencies for three microsatellite alleles in wild and farmed samples of *Haliotis midae* in south Africa. Number of individuals in parentheses.

Alleles (bp)	Dassen Is. (29)	Robben Is. (29)	Kleinmond (50)	Mossel Bay (50)	St. Francis (26)	Cape Recife (51)	West Farm (50)	East Farm (52)
			***CmrHr*2.15**					
243	-	-	0.031	-	-	0.020	-	-
249	-	0.058	-	0.017	-	-	-	-
251	-	-	-	-	-	0.010	-	-
253	-	0.019	0.010	0.017	-	0.020	0.061	0.010
257	-	0.019	0.010	-	-	-	0.010	-
261	-	0.038	0.052	-	-	-	0.031	-
263	-	0.058	-	-	-	-	0.010	-
265	0.024	-	0.052	-	-	-	-	-
267	0.286	0.308	0.365	0.103	0.229	0.176	0.306	0.354
269	0.071	0.038	-	-	-	-	0.020	-
271	-	-	0.010	-	-	-	-	-
273	0.048	-	0.010	-	-	0.039	-	-
275	0.071	-	0.010	-	-	0.020	0.020	0.031
276	0.024	-	-	-	-	-	-	-
277	0.452	0.385	0.333	0.690	0.625	0.569	0.398	0.531
279	-	0.019	0.031	0.086	0.021	0.069	0.082	-
281	0.024	0.038	0.063	0.069	0.063	0.069	0.010	0.042
283	-	-	0.021	0.017	0.063	0.010	-	0.021
293	-	0.019	-	-	-	-	-	-
303	-	-	-	-	-	-	-	0.010
309	-	-	-	-	-	-	0.051	-
			***CmrHr*2.23**					
243	0.917	0.904	0.878	0.897	0.808	0.843	0.898	0.830
253	0.083	0.096	0.122	0.103	0.192	0.157	0.102	0.170
			***CmrHr*2.29**					
426	0.354	0.212	0.214	0.310	0.558	0.314	0.300	0.610
428	-	-	0.010	0.034	-	0.010	-	-
430	-	-	0.010	-	-	-	-	-
442	-	-	0.010	-	-	-	-	-
444	-	-	0.010	-	-	-	-	-
446	-	-	0.010	-	-	-	-	-
448	0.063	0.096	0.071	-	0.038	0.069	0.070	0.040
450	-	-	0.010	-	-	0.010	0.030	-
452	-	-	0.020	-	-	-	0.010	-
454	0.021	-	0.010	-	-	-	0.050	-
456	-	-	0.020	-	-	-	-	0.010
458	0.021	-	0.010	0.086	-	0.029	0.030	-
460	-	-	0.031	0.069	-	-	-	-
462	0.438	0.615	0.500	0.345	0.365	0.451	0.440	0.310
464	0.021	-	0.020	-	-	0.010	0.020	-
466	-	-	0.020	0.017	-	-	0.010	-
468	0.083	0.077	0.020	0.138	0.038	0.108	0.040	0.030

Appendix C Allele frequencies at five microsatellite loci in farmed and natural samples of *H. rubra* Number of individuals in parentheses. GRK-Georges Rock, TRC-Trumpeter Corner, CUB-Curio Bay, OTP-One Tree Point, GTR-George Third Reef, STI-Sterile Island, NCR-Church Rocks, Race 1-Raceway 1, Race 2-Raceway 2, Race 3-Raceway 3, Race 4-Raceway 4, Farm-combined Raceway samples.

Allele (bp)	*GRK* (100)	*TRC* (61)	*CUB* (92)	*OTP* (93)	*GTR* (68)	*STI* (93)	*NCR* (96)	*Race 1* (64)	*Race 2* (64)	*Race 3* (64)	*Race 4* (64)	*Farm* (256)
					rubca							
110	0.015	0.016	0.012	0.033	0.008	-	0.036	-	-	-	0.031	0.008
114	-	-	0.006	-	-	-	-	-	0.024	-	-	0.006
116	-	-	-	-	-	-	-	-	0.008	-	-	0.002
118	0.305	0.246	0.285	0.255	0.164	0.293	0.286	0.117	0.143	0.127	0.164	0.138
120	-	-	-	-	-	-	-	-	0.032	-	-	0.008
122	0.025	0.008	0.006	0.022	-	0.017	0.005	0.156	0.008	-	0.063	0.057
124	-	-	-	-	-	-	-	-	0.349	-	-	0.087
126	0.020	0.025	0.006	0.016	0.008	-	0.031	-	0.016	-	-	0.004
128	0.005	0.016	-	-	0.016	0.011	0.021	-	-	-	-	-
130	-	-	0.006	-	-	-	-	0.023	-	-	-	0.006
132	-	0.008	-	0.005	-	-	0.005	-	-	-	-	-
134	0.015	0.008	0.012	0.011	0.016	0.023	0.021	-	-	0.024	0.094	0.030
136	0.005	0.008	-	0.005	-	0.011	-	-	-	-	-	-
138	0.015	0.008	0.017	0.005	0.025	0.011	0.005	-	-	-	0.070	0.018
140	-	0.008	-	0.016	0.008	0.017	-	-	-	0.206	-	0.051
142	0.045	0.008	0.029	0.011	0.049	0.034	0.021	-	-	0.016	0.016	0.008
144	0.065	0.066	0.041	0.022	0.082	0.034	0.052	-	0.008	0.016	0.094	0.030
146	0.010	-	0.017	0.011	0.008	0.006	0.010	-	-	-	-	-
148	0.020	0.025	0.023	0.054	0.025	0.040	0.036	0.023	-	0.008	0.055	0.022
150	0.015	0.008	0.017	0.005	0.016	0.017	0.026	-	0.008	-	-	0.002
152	0.020	0.049	0.047	0.082	0.074	0.052	0.042	0.008	-	0.008	0.023	0.010
154	0.005	0.033	0.017	0.011	0.025	0.011	0.005	-	0.016	-	-	0.004
156	0.030	0.049	0.029	0.038	0.049	0.046	0.026	-	0.167	0.032	-	0.049
158	0.105	0.09	0.093	0.098	0.057	0.080	0.052	-	0.024	0.405	0.055	0.120
160	0.035	0.074	0.041	0.033	0.090	0.040	0.052	0.008	0.167	0.008	0.047	0.057
162	0.015	0.041	0.047	0.033	0.057	0.029	0.052	0.016	0.032	0.016	0.031	0.024
164	0.030	0.041	0.070	0.098	0.041	0.046	0.063	0.016	-	-	0.070	0.022
166	0.025	0.016	0.029	0.022	0.049	0.017	0.021	-	-	-	-	-
168	0.035	0.008	0.052	0.027	0.033	0.052	0.031	-	-	-	-	-
170	0.020	0.041	0.017	0.011	0.008	0.011	0.016	-	-	0.135	0.016	0.037
172	0.005	0.008	0.012	0.016	0.025	0.006	0.010	-	-	-	0.055	0.014
174	0.025	0.025	0.006	0.016	0.016	0.023	0.026	-	-	-	-	-
176	0.020	0.016	0.012	0.011	0.008	0.023	0.005	0.203	-	-	0.047	0.063
178	0.005	-	0.006	0.016	0.008	0.006	0.021	0.242	-	-	-	0.061
180	0.010	0.016	0.006	-	-	0.011	-	0.188	-	-	-	0.047
182	-	-	-	0.005	-	0.006	-	-	-	-	-	-
184	-	0.008	-	-	-	0.006	0.010	-	-	-	0.008	0.002
186	0.005	-	-	-	-	-	-	-	-	-	-	-
188	0.005	-	0.006	-	-	0.006	-	-	-	-	-	-
190	0.015	-	0.006	-	-	-	0.005	-	-	-	-	-
192	0.015	0.008	-	0.005	0.016	0.011	0.005	-	-	-	-	-

Allele (bp)	*GRK* (100)	*TRC* (61)	*CUB* (92)	*OTP* (93)	*GTR* (68)	*STI* (93)	*NCR* (96)	*Race 1* (64)	*Race 2* (64)	*Race 3* (64)	*Race 4* (64)	*Farm* (256)
					***rubca* cont...**							
194	0.005	0.008	0.017	-	0.016	-	-	-	-	-	-	-
196	0.010	0.008	-	-	-	-	-	-	-	-	0.063	0.016
198	-	-	0.006	-	-	-	-	-	-	-	-	-
202	-	-	0.006	-	-	-	-	-	-	-	-	-
208	-	-	-	0.005	-	-	-	-	-	-	-	-
					***CmrHr* 1.14**							
251	0.060	0.066	0.044	0.060	0.113	0.083	0.089	-	0.008	0.195	0.070	0.068
259	0.760	0.779	0.874	0.832	0.766	0.800	0.768	0.961	0.547	0.773	0.906	0.797
261	0.095	0.139	0.071	0.076	0.073	0.089	0.105	0.039	0.438	0.031	0.008	0.129
263	0.020	-	-	0.011	-	0.006	-	-	-	-	-	-
265	-	-	-	0.005	0.008	-	-	-	-	-	-	-
267	0.030	0.008	0.011	0.011	0.016	0.011	0.011	-	-	-	0.008	0.002
269	0.010	-	-	-	-	0.006	0.016	-	-	-	-	-
271	0.005	-	-	-	0.024	-	-	-	-	-	-	-
275	0.010	-	-	-	-	-	-	-	-	-	-	-
277	-	0.008	-	-	-	0.006	-	-	-	-	-	-
283	-	-	-	0.005	-	-	-	-	-	-	-	-
289	0.010	-	-	-	-	-	0.005	-	-	-	0.008	0.002
291	-	-	-	-	-	-	-	-	0.008	-	-	0.002
293	-	-	-	-	-	-	0.005	-	-	-	-	-
					***CmrHr* 1.24**							
212	-	-	0.006	-	-	-	-	-	-	-	-	-
216	0.005	0.008	0.011	0.027	-	0.011	0.016	0.008	-	-	-	0.002
220	0.005	-	-	-	0.008	-	-	-	-	-	-	-
222	0.815	0.762	0.800	0.824	0.894	0.826	0.797	0.836	1.000	0.984	0.883	0.926
224	0.090	0.131	0.106	0.115	0.061	0.103	0.146	0.016	-	0.008	0.063	0.021
226	0.060	0.074	0.044	0.011	0.030	0.043	0.036	-	-	-	0.047	0.012
228	0.025	0.025	0.028	0.022	0.008	0.005	0.005	0.117	-	0.008	0.008	0.033
230	-	-	-	-	-	-	-	0.023	-	-	-	0.006
236	-	-	0.006	-	-	0.011	-	-	-	-	-	-
					***CmrHr* 2.14**							
200	-	-	-	-	0.016	-	-	-	-	-	-	-
208	0.016	-	0.007	-	0.016	0.006	0.011	-	-	-	-	-
212	0.032	0.025	0.049	0.056	0.016	0.056	0.011	-	-	0.024	0.024	0.012
216	0.112	0.107	0.090	0.099	0.081	0.112	0.087	0.039	0.211	0.056	0.119	0.106
220	-	0.016	-	0.037	0.016	0.006	0.005	-	0.008	0.008	-	0.004
224	0.537	0.459	0.521	0.420	0.419	0.438	0.489	0.563	0.531	0.603	0.365	0.516
228	0.165	0.205	0.153	0.247	0.274	0.206	0.179	0.367	0.070	0.294	0.254	0.246
232	0.011	0.008	0.007	0.019	0.016	0.025	0.043	0.008	0.008	0.016	-	0.008
236	0.128	0.180	0.174	0.123	0.145	0.138	0.174	0.023	0.172	-	0.230	0.106
240	-	-	-	-	-	-	-	-	-	-	0.008	0.002
252	-	-	-	-	-	0.013	-	-	-	-	-	-

Allele (bp)	*GRK* (100)	*TRC* (61)	*CUB* (92)	*OTP* (93)	*GTR* (68)	*STI* (93)	*NCR* (96)	*Race 1* (64)	*Race 2* (64)	*Race 3* (64)	*Race 4* (64)	*Farm* (256)
					***CmrHr*2.30**							
261	-	-	0.017	-	-	-	0.005	-	-	-	-	-
263	-	-	0.006	-	-	-	-	-	-	-	-	-
267	-	-	-	-	-	0.006	-	-	-	-	-	-
269	0.005	-	0.006	-	-	0.013	0.005	-	-	-	-	-
271	0.005	-	-	-	-	-	-	-	-	-	-	-
273	-	-	-	-	-	-	0.005	-	-	-	-	-
275	-	-	0.006	-	-	0.019	-	-	-	-	-	-
277	-	-	0.017	-	0.016	0.038	-	-	-	-	-	-
281	0.005	-	0.017	0.006	-	-	-	-	-	-	-	-
283	-	0.008	0.034	0.017	0.049	0.044	-	-	-	-	-	-
285	0.015	0.017	0.017	0.006	0.033	0.013	0.016	-	0.047	-	-	0.012
287	-	-	-	-	0.016	-	0.021	-	-	-	-	-
289	0.030	0.017	0.067	0.017	0.025	0.038	0.016	-	-	0.167	0.008	0.043
291	0.020	0.050	0.056	0.023	0.082	0.089	0.016	-	0.047	-	-	0.012
293	0.060	-	0.022	0.006	0.098	0.057	0.016	0.039	-	-	-	0.010
295	0.025	0.008	0.107	0.011	0.107	0.07	0.010	-	-	0.024	0.070	0.024
297	0.055	0.017	0.073	0.086	0.057	0.076	0.042	0.227	0.008	0.056	0.008	0.075
299	0.075	0.058	0.045	0.057	0.008	0.032	0.083	0.055	-	0.016	0.016	0.022
301	0.055	0.092	0.067	0.115	0.057	0.013	0.104	-	-	0.008	0.063	0.018
303	0.070	0.067	0.006	0.057	0.025	0.025	0.068	-	-	-	0.031	0.008
305	0.025	0.100	0.090	0.092	0.057	0.025	0.057	0.008	0.383	0.071	0.063	0.131
307	0.035	0.033	0.022	0.029	0.025	0.038	0.057	0.016	-	0.016	0.023	0.014
309	0.035	0.075	0.017	0.023	0.025	0.013	0.031	0.141	0.016	0.008	0.164	0.082
311	0.030	0.033	0.006	0.069	-	0.006	0.031	0.031	-	0.159	0.031	0.055
313	0.030	0.092	0.006	0.034	-	0.006	0.047	0.016	0.258	0.016	-	0.073
315	0.030	0.033	0.017	0.006	0.008	0.063	0.010	-	-	0.008	-	0.002
317	0.030	0.008	0.051	0.034	0.025	0.006	0.031	0.008	-	-	0.172	0.045
319	0.010	0.025	0.017	0.011	0.049	0.089	-	-	-	-	-	-
321	0.010	0.008	0.006	0.011	-	0.013	0.005	-	-	0.008	0.086	0.024
323	0.035	0.025	0.017	0.029	0.025	0.019	0.010	-	-	-	-	-
325	0.030	-	0.006	0.017	0.008	0.006	0.026	-	-	-	-	-
327	0.020	0.025	-	0.034	-	0.006	0.042	-	-	-	0.016	0.004
329	0.040	0.008	0.017	0.040	0.008	0.013	0.016	-	-	0.262	0.055	0.078
331	0.005	-	-	0.006	-	-	-	-	-	0.159	0.023	0.045
333	0.010	-	0.006	0.011	-	0.006	0.005	0.211	-	-	-	0.053
335	0.005	-	0.011	0.006	0.008	-	0.005	-	-	-	0.047	0.012
337	0.010	-	-	0.029	-	0.038	0.010	0.008	0.234	0.008	-	0.063
341	0.010	0.017	0.006	0.011	-	0.006	0.016	-	-	-	-	-
343	0.010	0.017	0.006	0.006	0.008	0.013	0.021	0.016	-	-	0.008	0.006
345	0.005	0.025	0.011	-	0.008	0.006	0.010	-	-	-	-	-
347	0.010	-	0.011	-	-	0.013	0.005	-	-	-	-	-
349	0.005	0.008	-	0.006	-	-	0.005	-	-	-	0.031	0.008
351	0.005	0.017	0.006	0.006	0.016	0.006	0.016	-	-	-	-	-
353	0.010	-	0.006	0.006	0.025	0.019	-	0.055	-	-	0.008	0.016

Allele (bp)	*GRK* (100)	*TRC* (61)	*CUB* (92)	*OTP* (93)	*GTR* (68)	*STI* (93)	*NCR* (96)	*Race 1* (64)	*Race 2* (64)	*Race 3* (64)	*Race 4* (64)	*Farm* (256)
					***CmrHr*2.30**		**cont..**					
355	0.005	0.008	-	0.006	-	0.013	0.010	0.008	-	-	0.008	0.004
357	0.005	0.008	0.017	-	0.016	0.013	0.005	-	0.008	0.008	0.023	0.010
359	0.010	-	0.017	0.006	-	-	0.005	-	-	-	-	-
361	0.005	0.017	-	0.011	0.016	-	0.016	-	-	-	-	-
363	0.010	-	-	0.023	0.008	-	0.016	-	-	-	-	-
365	0.015	0.025	-	-	-	0.006	0.021	-	-	-	-	-
367	0.010	0.008	0.022	-	0.008	-	0.010	0.008	-	0.008	0.047	0.016
369	0.005	-	0.028	-	-	-	0.005	-	-	-	-	-
371	0.015	-	-	0.017	-	0.006	-	-	-	-	-	-
373	0.020	0.008	-	-	0.025	-	0.010	-	-	-	-	-
375	0.005	-	0.011	0.006	0.008	0.006	0.010	-	-	-	-	-
377	-	0.025	-	0.006	-	0.006	0.010	-	-	-	-	-
379	0.025	-	0.011	0.006	0.008	-	0.005	-	-	-	-	-
381	-	-	-	-	0.016	-	-	0.156	-	-	-	0.039
385	0.005	0.017	-	-	0.008	0.006	0.010	-	-	-	-	-
389	-	-	-	-	0.016	-	-	-	-	-	-	-